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**14. ABSTRACT**

Doxorubicin (DOXO), cisplatin (CDDP) and paclitaxel (PAC) are most commonly used chemotherapeutic drugs for p53 mutant TNBC; however, the treatments eventually fail due to acquired drug resistance and toxicity. Here we report on the reconstitution of p53 in a p53-independent manner via p73 as an exciting possibility for improving cancer therapy. -TEA, an analog of vitamin E, which exhibits potent anticancer actions in vitro and in vivo, cooperates with DOXO or CDDP to enhance anticancer actions detected by apoptosis in p53 mutant TNBC cell lines. The combination treatments of -TEA with DOXO or CDDP enhanced p73 protein expression, enhanced levels of death receptors-5 (DR5), CD95/APO-1 (Fas), Bax and Noxa proteins, and reduced anti-apoptotic Bcl-2 protein levels, all of which are p53 regulated genes. The combination treatments induced enhanced levels of phospho-c-Ab1 and -JNK, as well as induced Yap nuclear translocation, which are upstream events for p73 activation. Data demonstrated that -TEA cooperates with chemotherapeutic drugs (DOXO and CDDP) to induce apoptosis via targeting p53 downstream apoptotic mediators in a p73-dependent manner. Furthermore, in vivo data show that -TEA inhibits DOXO-induced lung metastasis. Taken together, our in vitro and in vivo data suggest that the combination of -TEA plus clinically relevant chemotherapeutic drugs enhances anticancer actions in p53 mutant TNBC cells via activation of p73.

**15. SUBJECT TERMS**
Triple negative breast cancer, mutant p53, p73, -TEA, drug resistance, cisplatin, doxorubicin, apoptosis, metastasis

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## INTRODUCTION

Successful treatment of triple negative (ER-, PR-, HER2-) breast cancers (TNBC) that are also p53 mutant remains elusive. Unfortunately, the anticancer efficacy of commonly used chemotherapeutic agents, including doxorubicin (DOXO), cisplatin (CDDP) and paclitaxel (PAC) are limited due to acquired drug resistance and toxicities [1, 2]. Goals of this proposal were to investigate the *in vitro* and *in vivo* anticancer actions of combinations of chemotherapeutic agents plus RRR-α-tocopherol ether-linked acetic acid analog (α-TEA), a non-hydrolyzable ether analog of RRR-α-tocopherol in p53 mutant TNBC cells, and to understand the molecular signaling pathways involved in these events. Data, for the first time, show that α-TEA acts cooperatively with DOXO or CDDP to induce apoptosis via targeting p73 mediated p53-dependent apoptotic genes, which are regulated by c-Ab1, JNK and Yap pathways. *In vivo* data show that α-TEA inhibited DOXO-induced lung metastasis.

## BODY

Objective is to test the hypothesis that treatment of p53-mutant, triple-negative breast cancer (TNBC) cells with a unique analog of vitamin E (alpha-tocopherol ether-linked acetic acid analog; abbreviated α-TEA) in combination with chemotherapeutic agents will yield significantly better anticancer outcomes.

### Specific Aim 1. Evaluation of the antitumor actions of chemotherapeutic agents alone and in combination with α-TEA *in vitro* and *in vivo* in p53 mutant TNBC cells

### Task 1. *In vitro* clonogenic assessment of impact on survival of p53 mutant TNBC cells treated with chemotherapeutic agents alone and in combination with α-TEA

#### Methodology

*Cell culture* Human p53 mutant TNBC cell lines MDA-MB-231, BT-20 and MDA-MB-468 cells were purchased from the American Type Culture Collection (Manassas, VA). MBA-MD-231 and BT-20 cell lines were cultured in MEM media with 10% FBS and MDA-MB-468 cells were cultured in DMEM media with 10% FBS. For experiments, FBS was reduced to 2% to better mimic low *in vivo* serum exposure and cells were allowed to attach overnight before treatments. α-TEA (made in house) was dissolved in ethanol at 40 mM as stock solution. Equivalent levels of ethanol were used as vehicle (VEH) control for α-TEA. DOXO and CDDP (Sigma) were dissolved in $H_2O$.

*Colony formation assay*. Effects of treatments on colony formation were determined. Cells were seeded at 200 or 400 cells/well in 12-well plates and incubated overnight followed by treatments of the cells with different concentrations of α-TEA, DOXO, CDDP and PAC, as well as vehicle control for 12 days. Cells were washed with PBS, fixed with methanol and stained with 0.1% methylene blue in PBS. Colony forming cells were

expressed as cell survival (%) using control as 100%. $IC_{50}$ (half maximal inhibitory concentration) for inhibition of colony formation was calculated using CalcuSyn software (Biosoft, Cambridge, UK).

*Quantification of apoptosis.* Apoptosis was quantified by Annexin V-FITC/PI assay [Appendix].

*Western blot analyses.* Whole cell protein lysates were prepared and western blot analyses were conducted as described previously [Appendix].

**Results**

(i). Chemotherapeutic drugs alone and in combination with α-TEA exhibit *in vitro* antitumor actions in TNBC, p53 mutant cells

Colony formation and apoptosis assays were performed to evaluate *in vitro* antitumor actions of chemotherapeutic agents alone and in combinations with α-TEA in TNBC, p53 mutant cell lines; MDA-MB-231, BT-20, and MDA-MB-468. DOXO, CDDP and PAC inhibit colony formation and induce apoptosis in p53 mutant TNBC cells in a dose-response manner. The half maximal inhibitory concentrations ($IC_{50}$) of colony formation were 26 nM, 4.9 μM and 4 nM in MDA-MB-231 cells for DOXO, CDDP and PAC, respectively (BT-20 and MDA-MB-468 did not form good colonies in this study). The half maximal effective concentration ($EC_{50}$) for apoptosis is depicted in Table 1 (Published data, appendix). Different breast cancer cell lines exhibit differential sensitivity to chemotherapeutic drugs. Data show that MDA-MB-468 cells exhibit the most sensitive phenotype, and MDA-MB-231 cells exhibit the most resistant phenotype to DOXO, CDDP and PAC detected by apoptosis among the three tested cell lines. Antitumor effects of chemotherapeutic drugs + α-TEA were evaluated by colony formation and apoptosis assays. The combination index (CI) was calculated using Calcusyn software (Biosoft, Cambridge, UK). CI value < 1.0, CI value = 1, and CI value > 1 indicate synergistic, additive and antagonistic effects of combination, respectively. Data show that combinations of DOXO, CDDP or PAC plus α-TEA synergistically inhibited colony formation in MDA-MB-231 cells with CI values at 0.38, 0.46 and 0.36, respectively. Based on $EC_{50}$ of apoptosis, MDA-MB-231 and BT-20 cell lines, which are more resistant to DOXO and CDDP, were chosen to study the combination effects of α-TEA plus DOXO or CPPD on induction of apoptosis. α-TEA at 10 and 20 μM enhanced DOXO and CDDP induced apoptosis with CI values at 0.41 and 0.53 for DOXO, and 0.45 and 0.75 for CDDP in MDA-MB-231 and BT-20 cells, respectively (Published data, appendix). These data show that combinations of α-TEA + DOXO or CDDP synergistically induced apoptosis in TNBC, p53 mutant cells.

**Table 1. $EC_{50}$ for apoptosis**

| | DOXO (μM) | CDDP (μM) | PAC (nM) |
|---|---|---|---|
| MDA-MB-231 | 46.5 | 70.8 | 83.3 |
| BT-20 | 25.7 | 64.0 | 42.4 |
| MDA-MB-468 | 8.5 | 40.7 | 40.2 |

Furthermore, data showed that the combination of DOXO or CDDP plus α-TEA enhanced cleavage of caspases-8&9 and PARP in MDA-MB-231 and BT-20 cells, suggesting that the combination treatments induce caspase-8 and caspase-9 dependent apoptosis (Published data, appendix).

In summary, task 1 data demonstrated that α-TEA in combination with chemotherapeutic drugs exhibits cooperative *in vitro* antitumor actions in TNBC, p53 mutant cells.

**Task 2. *In vivo* therapeutic evaluation of impact on antitumor actions of p53 mutant TNBC cells treated with chemotherapeutic agent alone and in combination with α-TEA using a xenograft mice model**

**Methodology**

*Xenograft study*. Animal experiment was conducted according to `Guidelines for the Humane Treatment of Animals' as designated by the University of Texas Institutional Animal Care and Use Committee. Immune incompetent Nu/Nu female BALB/c mice at 5 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were injected subcutaneously with a total of $2 \times 10^6$ MDA-MB-231 human breast cancer cells in 100 μl media containing 50% matrigel (BD Biosciences, Franklin Lakes, NJ) in the inguinal area at a point equal distant from the fourth and fifth nipples on the right side. 10 days after tumor cell injection, the mice were randomly assigned to 4 groups (10 mice/group): control, α-TEA, DOXO and α-TEA + DOXO. α-TEA was delivered by diet at 500 mg/kg and DOXO was delivered by IP at 2 mg/kg once a week. AIN-76A diet was used for both control and α-TEA diets, which were purchased from Harlan Teklad (Madison, WI, USA). Tumors were measured every other day, and tumor volumes were calculated using the formula: volume ($mm^3$) = (width × width × length/2). Body weights were determined weekly.

*Tumor immunohistochemistry.* Immediately upon collection, tumors were fixed in formalin for immunohistochemical analyses. Deparaffinized 5 μm sections of tumor tissue (5 tumors/group) were examined for apoptosis and cell proliferation using reagents supplied in the ApopTag in situ Apoptosis Detection kit (Intergen, Purchase, NY, USA), and antibodies specific for nuclear antigen Ki-67 (DAKO Corp, Carpinteria, CA, USA) [3]. Brown TUNEL stained nuclei were scored as positive for apoptosis in 15 microscopic fields (400 ×)/tumor. Ki-67 positive stained cells were counted in 20 fields (400 ×)/tumor.

*Bouins solution for evaluation of lung metastases.* Lungs were excised, fixed and stained with Bouins solution, and destained with 70% ethanol. The number of lung lesions was determined under microscope.

*Statistical analyses*. Tumor growth was analyzed using one-way analysis of variance (ANOVA) with TUKEY's post-hoc test using Prim software version 4.0 (Graphpad, San Diego, CA). Differences in number of TUNEL and Ki-67 positive cells between control and treatments were determined with *t*-test using Prism software version 4.0 (Graphpad, San Diego, CA). A level of $P<0.05$ was regarded as statistically significant.

To test the anticancer potential of combinational treatments *in vivo*, immune deficient nu/nu mice were transplanted with MDA-MB-231 cells, the most resistant cell line to chemotherapeutic drugs tested in task 1. DOXO and α-TEA alone, and in combination were tested for their ability to inhibit tumor growth and lung metastasis. Tumor volumes in α-TEA, DOXO, and combination groups were reduced in comparison with control; however, the tumor volumes in α-TEA + DOXO combination group had no difference in comparison with α-TEA and DOXO alone. Although α-TEA and DOXO reduced tumor volume there is no statistical difference in comparison with control. There are no statistical differences of TUNEL and Ki67 positive cells, the markers for apoptosis and proliferation, respectively, between control and treatments (Data not shown). These results are unexpected based on our and other group's data showing that α-TEA significantly inhibited tumor volume and tumor cell proliferation, as well as induced tumor cells to undergo apoptosis in different cell types and mice models [3, 4, 5, 6, 7]. A possible explanation for not obtaining expected results with α-TEA alone comes from post-study analyses of the commercially purchased defined diet pellets supplemented with α-TEA showing that α-TEA was not evenly distributed in the pellets. However, when the study was repeated by delivery of α-TEA by gavage formulated with liposome we obtained results for α-TEA that were similar to the first animal study in that tumor volumes were reduced in comparison with control, but the data were not statistically different compared with control. Combination of DOXO + α-TEA slightly reduced tumor volumes in comparison with single treatments alone, but data was not statistically different. Future studies using higher levels of α-TEA may be needed to resolve these differences.

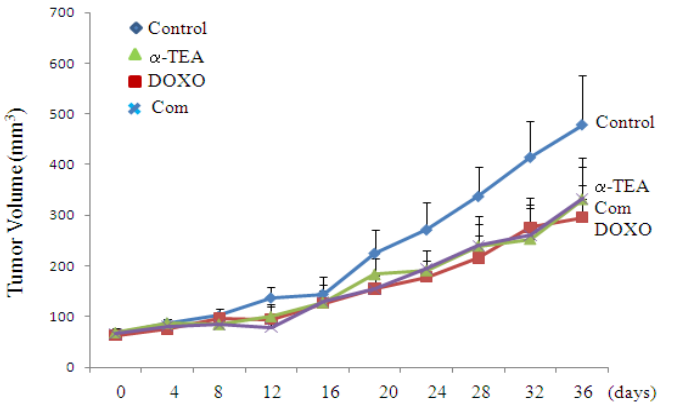


**Figure 1 legend. Evaluation of tumor growth.** Supplemented diets were initiated at day 0 which was 10 days after tumor cell injections. Tumor volumes ($mm^3$) are depicted as mean + S.E.

Although α-TEA did not cooperate with DOXO to inhibit tumor growth, the combination treatment blocked DOXO-induced lung metastasis. DOXO alone increased incidence of visible lung metastasis (60%) in comparison with control (20%) (Table 2). There were no lung metastases in the α-TEA treated group (Table 2). Furthermore, α-TEA in combination with DOXO reduced DOXO-induced increase in incidence of lung metastasis from 60% to 40% (Table 2). The findings that α-TEA alone prevented

**Table 2. Ability of α-TEA to prevent DOXO-induced lung metastases**

| Treatments | macroscopic lung metastases (%)(1) | Average no. metastasis foci/mouse(2) |
|---|---|---|
| Control | 2 | 0.2 |
| α-TEA | 0 | 0 |
| DOXO | 6 | 0.9 |
| Combination | 4 | 0.4 |

(1) The percentage of the mice who were detected for metastasis foci from total 10 mice/group

(1) Average number of metastasis foci/mouse from 10 mice/group

lung metastases and that α-TEA in combination with DOXO reduced the ability of DOXO to induce lung metastases are important for treatment of triple negative breast cancer. Accumulating data support the concept that cancer stem cells, a sub-population in tumors, are responsible for tumor initiation, tumor development, metastasis and drug resistance, as well as recurrence [8, 9, 10]. Traditional chemotherapeutic drugs, such as DOXO, only target bulk tumor cells (non-cancer stem cells); however, maintains or increases numbers of cancer stem cells [11], leading to cancer recurrence, metastasis and drug resistance. Our data show that DOXO induces lung metastasis in triple negative breast cancer, suggesting that DOXO enhances numbers of cancer stem cells. Since chemotherapeutic drugs remain the only effective therapy for primary tumor of triple negative cancer, new strategies, such as α-TEA, that eliminate cancer stem cells are needed to prevent/reduce chemotherapy mediated recurrent tumors, which are highly metastasis and drug resistant. More in-depth studies are needed to further evaluate the combination effects of DOXO + α-TEA on the elimination of bulk as well as cancer stem cells.

**Specific Aim 2. Conduct mechanistic studies to determine the pathways used by α-TEA to synergize with chemotherapeutic agents to yield enhanced antitumor actions**

**Task 1. Determine if p73 is regulated by chemotherapeutic agents positively via activation of c-Abl/JNK and negatively via activation of Akt/pYap**

**Methodology**

RNA interference. Transfection of MDA-MB-231 cells with siRNA to p73, c-Abl, JNK, and Yap or control (Ambion, Austin, TX) was performed following the method previously described [Appendix].

**Results**

(i). The effects of c-Abl and JNK on chemotherapeutic drugs-induced p73 expression

Published data show that p73 is up-regulated in response to a subset of DNA-damaging agents, including DOXO, CDDP, camptothecin and etoposide [12]. Our data also show that DOXO and CDDP induce increased levels of p73 protein expression in p53 mutant TNBC cell lines (Data not shown). 73 is predominantly regulated at the post-translational level in response to DNA damaging agents. Both c-Abl and JNK are activated by DNA damaging agents and play an important role in p73 activation [13, 14]. DOXO and CDDP have been shown to regulate p73 via c-Ab1 [13, 15]. JNK is required for p73 mediated apoptosis via formation of a complex with p73 and phosphorylating p73 at several serine and threonine residues [14]. Besides direct phosphorylation of p73, JNK also regulates p73 via activation of c-Abl [16]. In normal cells, c-Abl is sequestered in the cytosol by 14-3-3 proteins. Upon exposure of cells to DNA damaging agents, JNK is activated and phosphorylates 14-3-3, resulting in the release of c-Abl into the nucleus, which is required for the induction of apoptosis in response to

DNA-damaging agents [16, 17]. Data also show that c-Abl regulates JNK [18], suggesting cross-talk between c-Abl and JNK in treatment activation of p73. Our data show that both DOXO and CDDP induce increased levels of p73 as well as pc-Abl at Tyr-245 and pJNK, and siRNAs to c-Abl and JNK blocked the ability of DOXO and CDDP to induce p73 protein expression in MDA-MB-231 cells (Fig 2). siRNAs to c-Abl and JNK blocked both DOXO and CDDP induced pc-Abl and pJNK protein expression**,** respectively. These data indicate that c-Able and JNK are upstream mediator of p73.

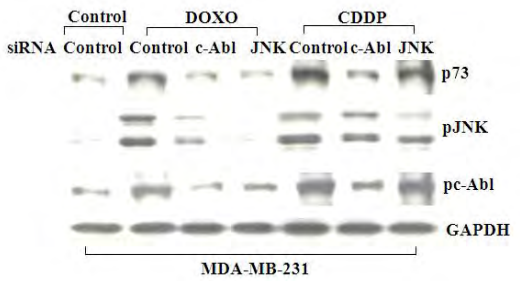


**Figure 2 legends. c-Abl and JNK are involved in DOXO and CDDP induced p73 up-regulation.** MDA-MB-231 cells were transfected with siRNAs to JNK, c-Abl and control for 2 days followed by treating the cells with DOXO or CDDP for 15 hrs. Western blot analyses were performed to determine the effects of the siRNAs on p73, pJNK, and pc-Abl protein expression with GAPDH as loading control. Antibody to p73 was purchased from (Imgenex).

(ii) Yap is involved in DOXO- and CDDP-induced up-regulation of p73

Yap is a transcriptional coactivator Yes-associated protein, which can interact with the p53 family member p73, resulting in an enhancement of p73's transcriptional activity and stability [17, 18, 19, 20]. A potential mechanism of the p73 protein stabilization was recently suggested by Levy that YAP1 competes with Itch, an E3 ubiquitin ligase involved in degradation of p73, for binding to p73 at the PPXY motif [21]. Yap activity can be regulated by c-Abl via phosphorylation at Tyr-357, leading to stabilization of Yap with higher affinity to p73, as well as Yap translocation into the nucleus [17, 22]. Furthermore, Yap can be negatively regulated by Akt. Akt induces Yap phosphorylation at ser-127, resulting in Yap cytosol localization via promoting Yap binding with 14-3-3 and inactivation of Yap [23]. Therefore, Yap activation can be regulated positively by c-Abl and negatively by Akt. DNA damage activates Akt via DNA-PKA [24]. Akt inhibitors have been reported to enhance the anti-cancer effect of doxorubicin [25]. Our data show that siRNA to Yap blocked DOXO and CDDP induced 73 up-regulation (Fig 3A), suggesting that Yap is involved in DOXO and CDDP induced activation of p73. To determine if Akt has a role in DOXO or CDDP induction of p73 we examined the effect of PI3K/Akt inhibitor wortmannin, DOXO and CDDP alone and in combination on p73 protein expression. Data show that PI3K inhibitor cooperates with DOXO and CDDP to induce p73 protein expression. DOXO and CDDP enhance pAkt and pYap protein expression and PI3K inhibitor inhibited it in MDA-MB-231cells (Fig

3B, published data, appendix). These data suggest that suppression of DOXO and CDDP induced increases in pAkt and pYap may enhance DOXO and CDDP induced activation of p73.

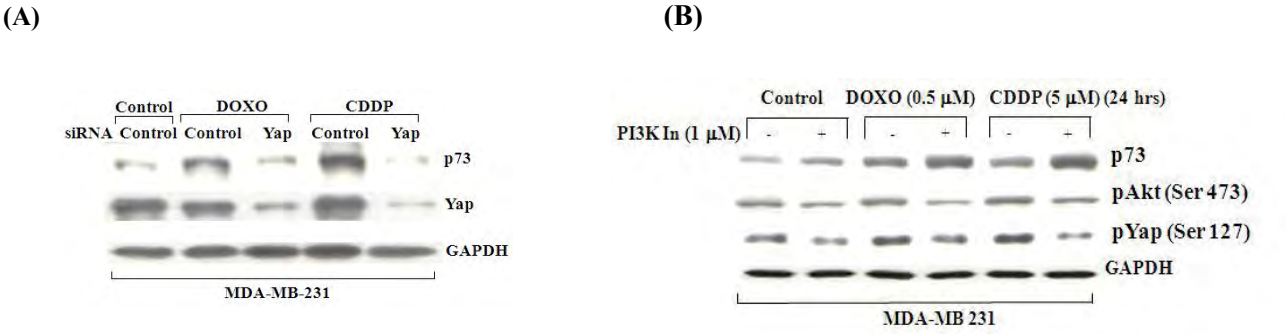


**Fig 3 legend.** MDA-MB-231 cells were transfected with siRNAs to Yap and control for 2 days followed by treating the cells with DOXO or CDDP for 15 hrs. Western blot analyses were performed to detect protein levels of p73 and Yap with GAPDH serving as loading control (A). MDA-MB-231 cells pre-treated with PI3K inhibitor wortmannin (Cell Signaling Technology) for 2 hrs, followed by treatment with DOXO or CDDP for 24 hrs. Western blot analyses were performed to detect protein levels of p73, pAkt and pYap with GAPDH serving as loading control (B).

## Task 2. Determine if α-TEA sensitizes cancer cells to chemotherapeutic agents by enhancing p73 expression via activation of c-Abl/JNK and suppression of Akt/pYap

### Methodology

*Nuclear and cytoplasmic fractionation.* Cytoplasmic and nuclear fractions were prepared as previously described [Appendix]

*Statistical Analysis.* Apoptosis data were analyzed for the statistical difference between treatments using student *t*-test. Differences were considered statistical significant at $p < 0.05$ [Appendix].

(i). p73 is upregulated by combinations of α-TEA plus DOXO or CDDP and involved in combination induced apoptosis

Since both DOXO and CDDP, as well as α-TEA, induce p73 upregulation in breast cancer cells [12, 26, Fig 2], the combination of α-TEA plus DOXO or CDDP was investigated for ability to cooperatively enhance p73 protein expression. Single treatments with DOXO, CDDP or α-TEA at sub-apoptotic levels slightly increased p73α protein expression; whereas, combinations of α-TEA + DOXO or α-TEA + CDDP at the same levels markedly enhanced p73 protein expression in comparison with single treatments in both MDA-MB-231 and BT-20 cells (Figure 4 A&B, published data, appendix). siRNA to p73 significantly reduced the ability of α-TEA + DOXO or α-TEA + CDDP to induce apoptosis as determined by Annexin V and PARP analyses in MDA-MB-231 cells (Figure 4 C&D, published data, appendix). Western blot data show that siRNA to p73 effectively silenced p73 protein expression (Figure 4 D). These data indicate that p73 activation by combination treatments is critical for induction of cell death by apoptosis.

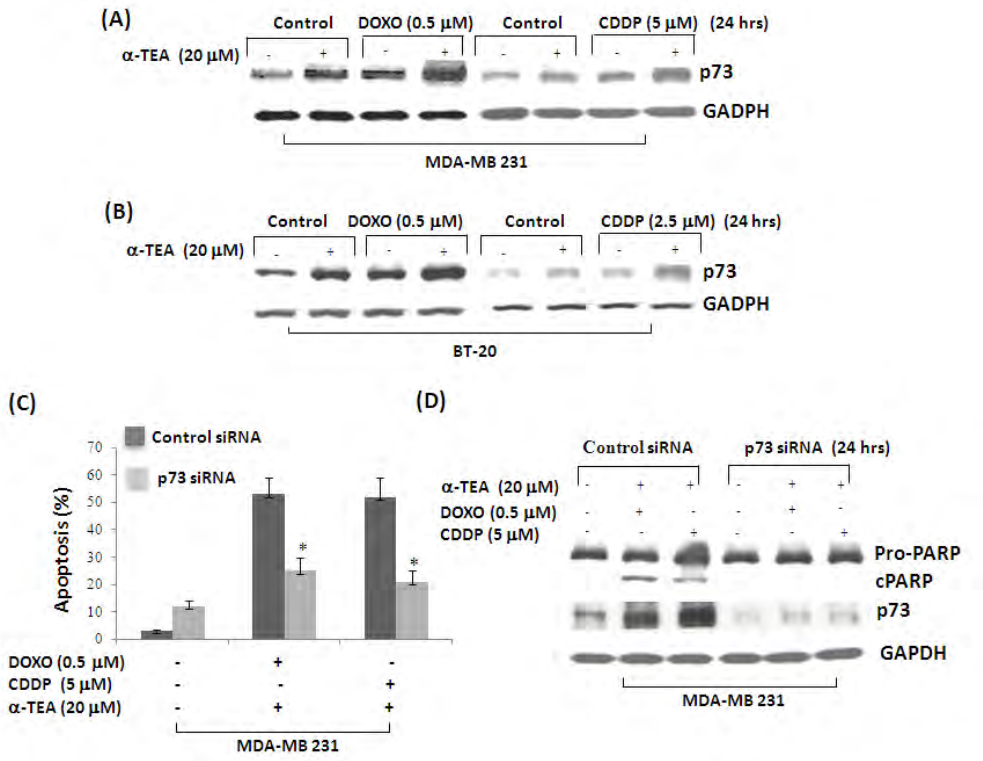


**Figure 4 legend. p73 is up-regulated by combination treatments and involved in combination induced apoptosis.** MDA-MB-231 and BT-20 cells were treated with α-TEA, DOXO and CDDP alone or in combination for 24 hrs. Western blot analyses were performed to detect protein levels of p73 with GAPDH serving as loading control (A&B). MDA-MB-231 cells were transfected with siRNAs to p73 or control for 2 days followed by treating the cells with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. FACS/Annexin V assay was used to determine the percentage of apoptotic cells (C). Western blot analyses were used to verify the knockdown efficiency of p73 siRNA and the effect of p73 siRNA on combination-induced PARP cleavage (D). *$p$<0.05 = significantly different from control siRNA determined by *t*-test.

(ii). α-TEA cooperates with DOXO or CDDP to upregulate phospho-c-Abl and -JNK, which are upstream mediators of p73

Our data in task 1 (Aim 2) show that p73 is up-regulated by DOXO and CDDP in MAD-MB-231 cells via activation of c-Abl and JNK (Fig 2). To understand how p73 is activated by the combination treatments, phosphorylated levels of c-Abl (Tyr-245) and JNK2/1 were examined. Combinations of α-TEA plus sub-apoptotic doses of DOXO or CDDP induced increased levels of phosphorylated c-Abl (Tyr-245, pc-Abl) and JNK2/1 (pJNK) in both MDA-MB-231 and BT-20 cancer cells (Figure 5A&B, published data, appendix). siRNAs to c-Abl and JNK reduced the ability of combination treatments to induce apoptosis as determined by Annexin V (Figure 5C, published data, appendix) and PARP cleavage (Figure 5D, published data, appendix). Knockdown of c-Abl and JNK significantly reduced levels of pc-Abl (Tyr-245) and pJNK 2/1 (Figure 5D) and also blocked the ability of combination treatments to increase the levels of p73 (Figure 5D). siRNA to c-Abl blocked the ability of combination treatments to induce increased levels of pJNK; whereas, siRNA to JNK has no effect on the ability of combination treatments to induce increased levels of pc-Abl (Figure 5D). These data show that activation of p73 is mediated by c-Abl and JNK in the combination treatments, and suggest that c-Abl regulates the phosphorylation status of JNK.

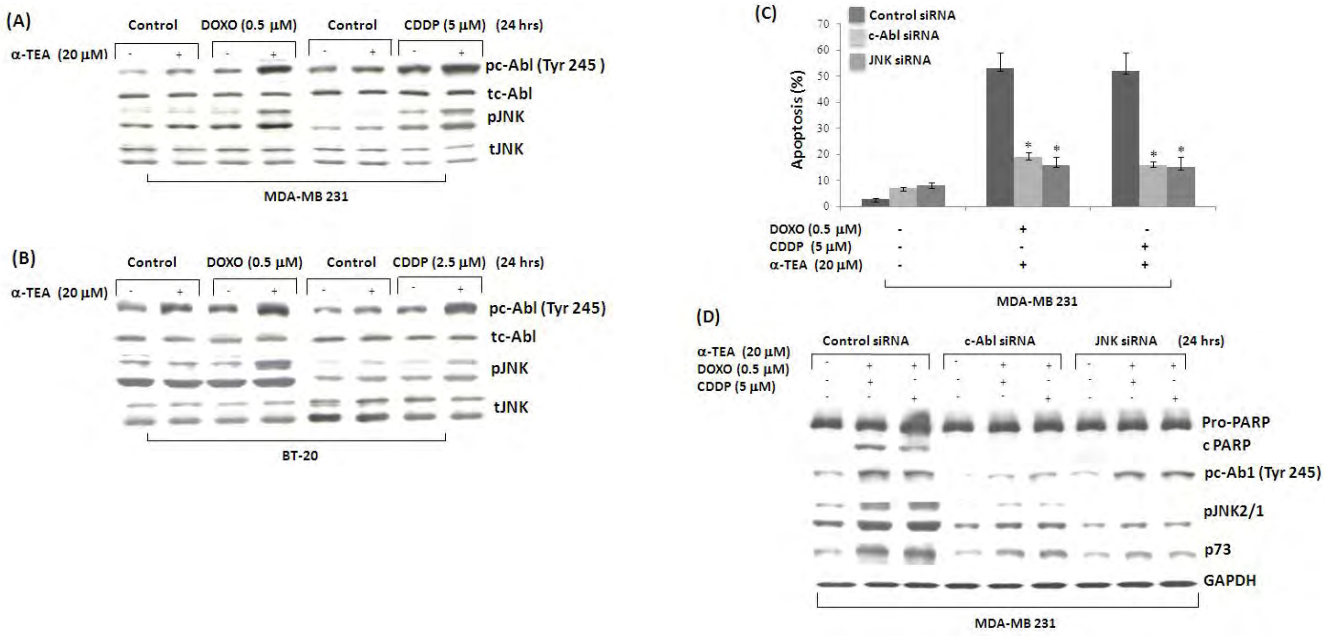


**Figure 5 legends. α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which can serve as upstream mediators of p73.** The protein levels of pc-Abl, total c-Abl (tc-Abl), pJNK1/2, and total JNK1 (tJNK1) were determined by western blot with GAPDH as loading control (A&B). MDA-MB-231 cells were transfected with siRNAs to c-Abl, JNK and control for 2 days and treated with combination of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. Apoptosis was determined by Annexin V/FACS (C). Western blot analyses were used to verify the knockdown efficiency of c-Abl and JNK siRNAs and the effect of c-Abl and JNK siRNAs on combination induced PARP cleavage, and p73 (D). **p*<0.05=significantly different from control siRNA determined by *t*-test.

(iii). Yap is activated by combinations of α-TEA with DOXO or CDDP and is involved in combination-induced apoptosis

Data in task 1 (Aim 2) show that Yap is involved in DOXO and CDDP induced up-regulation of p73 in MDA-MB-231 (Fig 3A). Here, studies were conducted to determine if Yap was involved in combination mediated p73 up-regulation. Since Yap can be activated by translocation into the nucleus [23], the effect of combination treatments on Yap translocation from the cytosol to the nucleus was investigated. Treatment of MDA-MB-231 cells with α-TEA + DOXO or α-TEA + CDDP induced increased levels of Yap protein in the nuclear fraction and reduced levels of Yap protein in the cytoplasmic fraction. Histone 1 and GAPDH were used to evaluate purity of nuclear and cytoplasmic fractions, respectively (Fig 6A, published data, appendix). siRNA knockdown of Yap markedly reduced levels of Yap and significantly reduced the ability of combination treatments to induce apoptosis as measured by annexin V analyses (Fig 6B, published data, appendix) and western blot analyses of PARP cleavage (Fig 6C, published data, appendix). Yap knockdown also markedly reduced the ability of combination treatments to increase levels of p73 (Fig 6C). These data show that YAP is a key player in combination treatment-induced apoptosis.

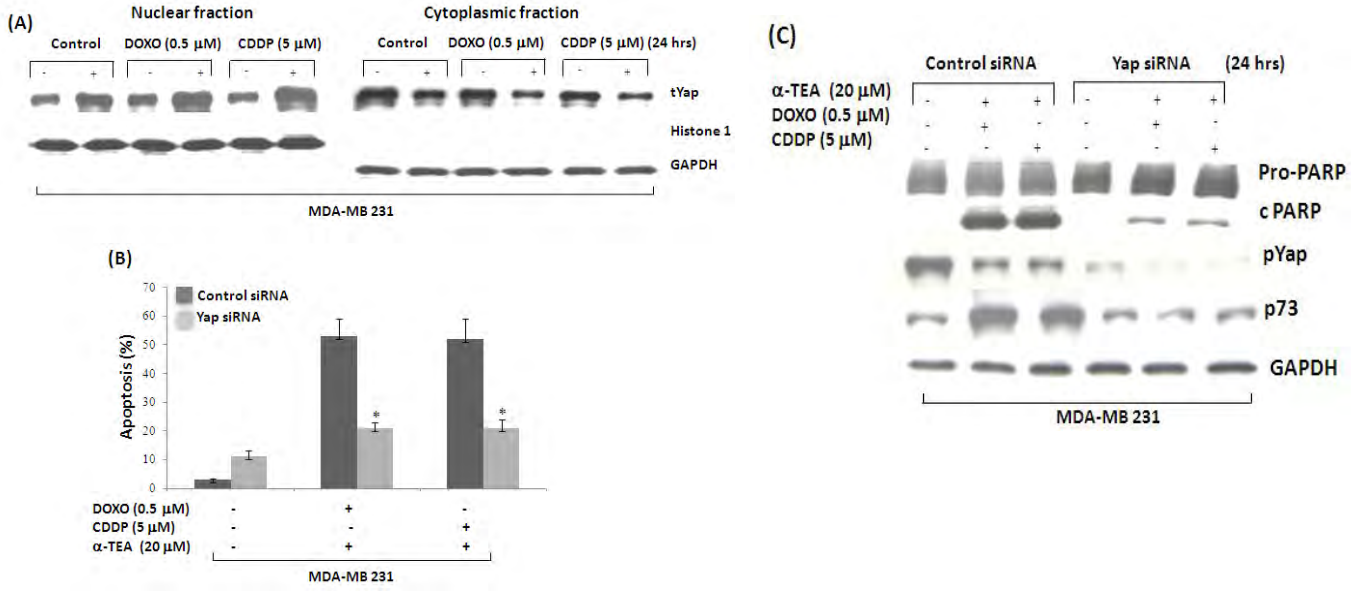


**Figure 6 legends. α-TEA cooperates with DOXO or CDDP to induce Yap translocation from cytosol to nuclear and Yap is involved in combination induced apoptosis and p73 activation.** Isolated cytosolic and nuclear fractions using the methods described in **Methodology "**Nuclear and cytoplasmic fractionation" in Aim 2 task 2 were used to detect Yap tanslocation from cytosol to the nucleus by western blot analyses. Antibody to Yap was purchased from Cell Signaling Technology (A). MDA-MB-231 cells were transfected with siRNAs to Yap and control for 2 days followed by treating the cells with combination of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. Apoptosis was determined by Annexin V/FACS (B). Western blot analyses were used to verify the knockdown efficiency of YAP siRNA and the effect of YAP siRNA on combination induced PARP cleavage and p73 (C). **p*<0.05=significantly different from control siRNA determined by *t*-test.

(iv). α-TEA cooperates with DOXO or CDDP to suppress phosphorylated Akt and Yap

Data in task 1 (Aim 2) show that inhibition of Akt using PI3K inhibitor induces p73 protein expression and decreased pYap (Ser-127) protein expression in MDA-MB-231 cells, suggesting that Akt plays a role in p73 protein expression via phsphorylation of Yap at Ser-127. Since α-TEA has been reported to decrease pAkt in prostate, ovarian and breast cancer cells [3, 27, 28], and combination treatments induces Yap nuclear translocation, we hypothesized that the combination of α-TEA plus DOXO or CDDP may decrease pAkt and pYap at ser-127. To test this hypothesis the combination treatments were investigated for effects on pAkt and pYap (Ser-127) expression. α-TEA cooperates with DOXO or CDDP to suppress pAkt (Ser-473) and pYap (Ser-127) in MDA-MB-231 (Fig 7, published data, appendix). DOXO and CDDP at sub-apoptotic doses slightly induced increased levels of pAkt and pYap (Fig 7). These data suggest a role for combination treatment to suppress pAkt (Ser-473) and pYap (Ser-127) in Yap nuclear translocation and p73 activation.

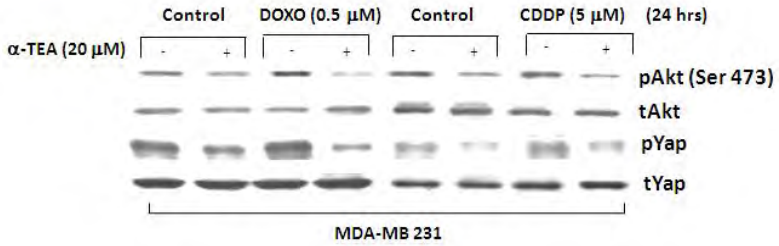


**Figure 7 legends. α-TEA cooperates with DOXO or CDDP to suppress pAkt and pYap protein expression.** MDA-MB-231 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. The protein levels of pAkt and pYap were determined by western blot with total Akt and Yap serving as controls.

## Task 3. Determine if p53 inducible genes (Fas, DR5, Bax and Noxa) are induced by p73 in α-TEA + chemotherapeutic drug-induced apoptosis in TNBC cells

### Methodology

RT-PCR detection of Fas, DR5, Bax, Noxa and Bcl-2 mRNA expression. Total RNA was extracted using RNA isolation kit (Qiagen Inc. Valencia, CA). Semi-quantitative analyses were conducted to detect Fas, DR5, Bax, Noxa and Bcl-2 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) using the housekeeping gene β-actin as control. The detail methods were described previously [Appendix].

(i). Combinations of α-TEA plus DOXO or CDDP upregulate DR5, Fas, Bax, and Noxa pro-apoptotic proteins and downregulate Bcl-2 anti-apoptotic protein

Published data show that p73 can regulate p53-dependent genes in p53-deficient cells [29]. To better understand the cellular events involved in p73 mediated apoptosis in combination treatments, mRNA and protein expression of p53-dependent apoptotic mediators Fas, DR5, Bax, Noxa, and anti-apoptotic mediator Bcl-2 were examined. Combinations of α-TEA plus DOXO or CDDP enhanced Fas, DR5, Bax and Noxa mRNA and protein expression, and decreased Bcl-2 mRNA and protein expression in MDA-MB-231 and BT-20 cells (Figure 8A, B, C, & D, published data, appendix).

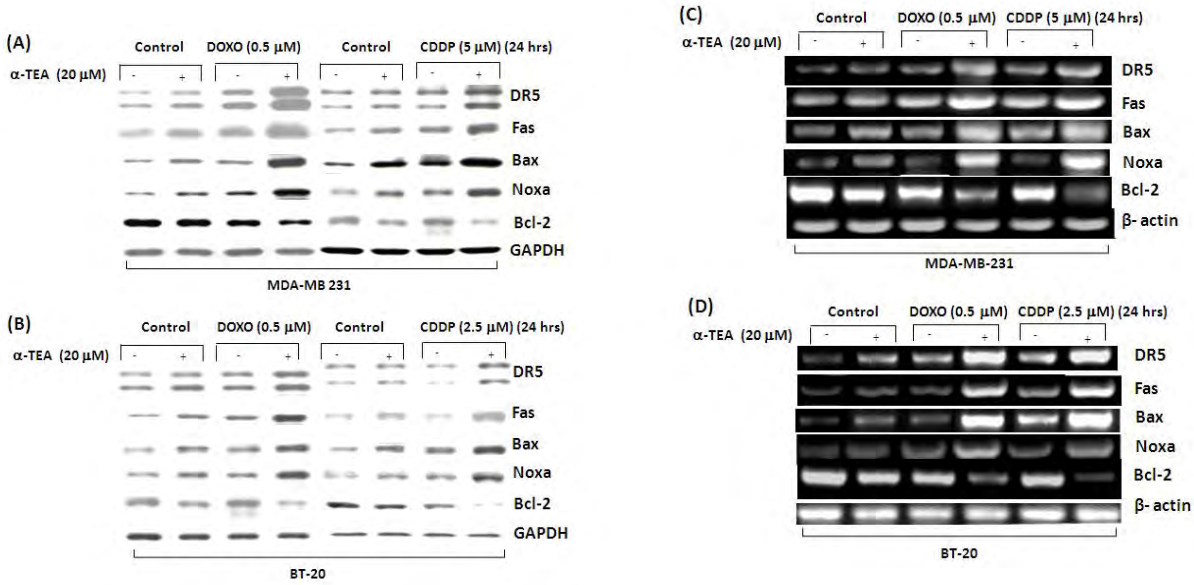

**Figure 8 legends. α-TEA cooperates with DOXO or CDDP to up-regulate DR5, Fas, Bax, and Noxa pro-apoptotic proteins and down-regulate Bcl-2 anti-apoptotic protein.** MDA-MB-231 and BT-20 cells were treated with α-TEA, DOXO or CDDP alone or in combination with α-TEA for 24 hrs. The protein levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by western blot analyses with GAPDH as loading control (A&B). mRNA levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by RT-PCR described in **Methodology "**RT-PCR detection of Fas, DR5, Bax, Noxa and Bcl-2 mRNA expression" with β-actin as loading control (C&D).

(ii). p53 downstream gene expressions are regulated by p73 in combination treatments of α-TEA + DOXO and α-TEA + CDDP

Although p73 has been reported to induce apoptosis via activation of p53 death signaling mediators in p53 mutant cells [30], p53 dependent apoptosis-related genes that are regulated by p73 in p53 mutant cancer cells have not been fully identified. To determine if the combination treatment mediated p53 downstream genes are regulated by p73, siRNA knockdown of p73 was performed. siRNA to p73 effectively silenced p73 protein expression and blocked the ability of combinations of α-TEA + DOXO or CDDP to induce increased levels of DR5, Fas, Bax and Noxa protein and decreased level of Bcl-2 protein (Figure 9A, published data, appendix). Our studies identified DR5, Fas, Bax, Noxa and Bcl-2 proteins as downstream mediators of p73 in triple negative, p53 mutant breast cancer cells. Taken together, these data demonstrated that combination treatments with α-TEA + DOXO or CDDP induce apoptosis in triple negative, p53 mutant breast cancer cells by up-regulation of apoptotic mediators Fas, DR5, Bax and Noxa, and by down-regulation of anti-apoptotic mediator Bcl-2 via p73.

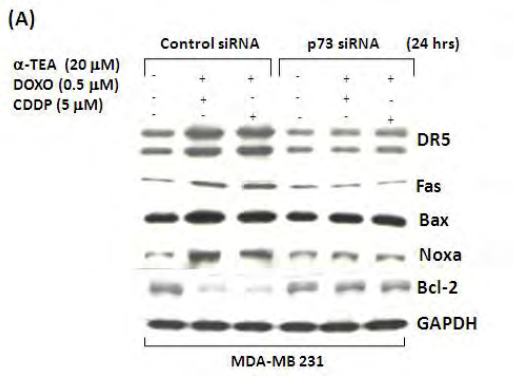


**Figure 9 legends. p53 downstream gene expressions are regulated by p73 in combination treatments.** MDA-MB-231 cells were transfected with p73 or control siRNAs for 2 days and treated with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. Western blot analyses were used to detect protein levels of DR5, Fas, Bax, Noxa and Bcl-2 with GAPDH as loading control.

**(iii). p53 downstream gene expressions are regulated by JNK, c-Abl and Yap in combination treatments of α-TEA + DOXO and α-TEA + CDDP**

Since JNK, c-Abl, and Yap are upstream mediators of p73 in combination treatments, we hypotheses that p53 downstream gene expressions are regulated by JNK, c-Abl and Yap in combination treatments. To confirm this hypothesis, siRNA knockdown of JNK, c-Abl and Yap was performed to determine if expression levels of p53 downstream genes are regulated by JNK, c-Abl and Yap. siRNA to JNK, c-Abl and Yap blocked the ability of combinations of α-TEA + DOXO or α-TEA + CDDP to induce increased levels of DR5, Fas, Bax and Noxa protein and decreased level of Bcl-2 protein (Figure 10A&B, published data, appendix). These data demonstrated that c-Abl, JNK and Yap can regulate p53 downstream genes DR5, Fas, Bax, Noxa and Bcl-2 via p73.

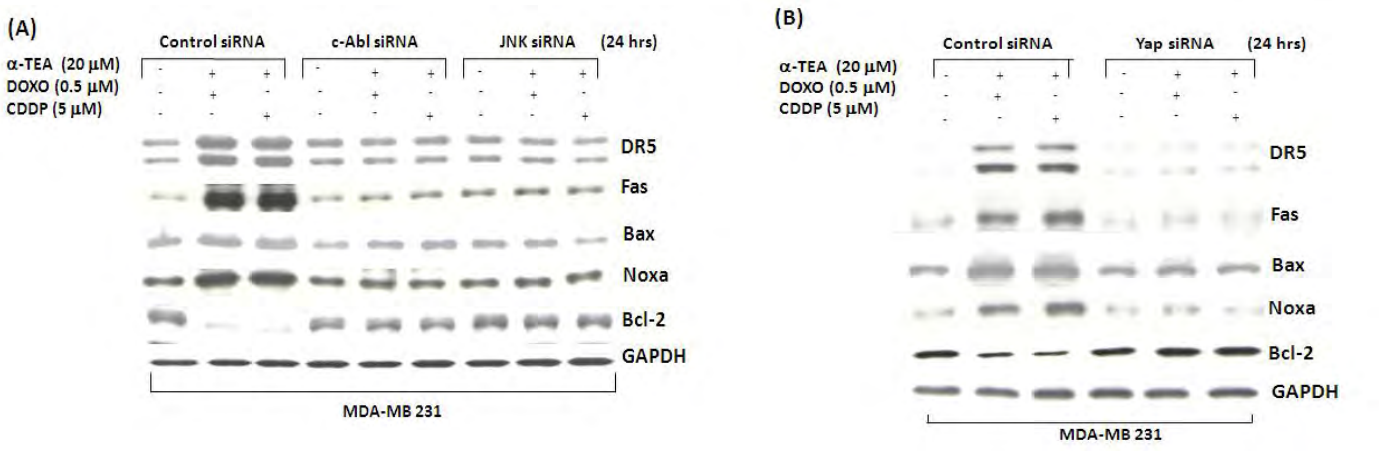


**Figure 10 legends. p53 downstream gene expressions are regulated by JNK, c-Abl and Yap in combination treatments.** MDA-MB-231 cells were transfected with siRNAs to JNK (A), c-Abl (A), Yap (B) or control for 2 days and treated with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. Western blot analyses were used to detect protein levels of p73-mediated DR5, Fas, Bax, Noxa and Bcl-2.

In summary, our data demonstrated that α-TEA, a small bioactive lipid, cooperates with DNA damaging agents DOXO or CPPD to induce apoptosis in triple negative, p53 mutant breast cancer cells via activation of p73, a promising target for breast cancers, especially for triple negative and p53 mutant breast cancers. In addition, α-TEA shows the potential to inhibit DOXO-induced lung metastasis in xenograft mouse model transplanted with MDA-MB-231 cells. Since cancer stem cells are responsible for metastasis and DOXO has the ability to stimulate cancer stem cells [11] we hypothesis that α-TEA inhibits DOXO-induced lung metastasis via eliminating cancer stem cells. One of the targets to eliminate cancer stem cells is let-7 miRNA [31], which can be regulated by p73 [32]. Thus, p73 activation in combination treatments may also contribute to combination effect of α-TEA + DOXO on inhibition of DOXO-induced lung metastasis, via elimination of DOXO-induced cancer stem cells.

## KEY RESEARCH ACCOMPLISHMENTS

- Chemotherapeutic drugs; DOXO, CDDP and PAC alone and in combination with α-TEA exhibit *in vitro* antitumor action in p53 mutant, TNBC cell lines.
- DOXO and CDDP alone and in combination with α-TEA induce p73 protein expression in p53 mutant TNBC.
- c-Abl/JNK and pAkt/YAP are involved in p73 protein expression in DOXO and CDDP alone and in combination with α-TEA treatments in p53 mutant TNBC cells.
- p53 downstream genes; Fas, DR5, Bcl-2, Bax, and Noxa are regulated by p73 in DOXO or CDDP combination treatments with α-TEA. c-Abl, JNK and YAP are involved in p53 downstream protein expression in p53 mutant TNBC.
- *In vivo* data show that α-TEA blocked DOXO-induced lung metastasis, suggesting the potential of α-TEA as an adjuvant to prevent/reduce DOXO mediated recurrence of triple negative breast cancer.

## REPORTABLE OUTCOMES

Publication:

Tiwary R, Yu W, Sanders BG, Kline K. α-TEA cooperates with chemotherapeutic agents to induce apoptosis of p53 mutant, triple-negative human breast cancer cells via activating p73. Breast Cancer Res 2011;13(1):R1. [PubMed: 21214929] [Appendix]

Oral presentation:

Richa Tiwary. Strategy for Restoring Drug Sensitivity to Human Breast Cancers (2009) Annual South Pardre Island Symposium on Advances in Cell Signaling, Cancer Prevention and Therapy, South Pardre Island, TX.

Abstracts:

Weiping Yu, Richa Tiwary, Bob G. Sanders and Kimberly Kline (2010) Targeting p73 as a strategy for enhancing anticancer efficiency of chemotherapeutic drugs to triple negative human breast cancers. 2010 AACR annual meeting, Washington, DC

Weiping Yu, Richa Tiwary, Bob G. Sanders and Kimberly Kline (2011) α-TEA cooperates with chemotherapeutic agents to induce apoptosis of p53-mutant, triple-negative human breast cancer cells via activating p73. 2011 DOD Era of Hope Conference, Orlando, FL.

## CONCLUSIONS

Goals of this study were to test the hypothesis that treatment of p53-mutant, triple-negative breast cancer (TNBC) cells with a unique analog of vitamin E (alpha-tocopherol ether-linked acetic acid analog; abbreviated α-TEA) in combination with chemotherapeutic agents would yield significantly better anticancer outcomes. Data generated by these studies demonstrated that combinations of α-TEA + chemotherapeutic agents; DOXO and CDDP enhance *in vitro* antitumor actions in TNBC and p53 mutant cells. The enhanced antitumor actions are regulated by p73 via targeting p53 downstream genes: Fas, DR5, Bax, Noxa and Bcl-2. Activation of c-Abl,

JNK and YAP plays important roles in regulation of p73 and p73 mediated p53 downstream proteins. In addition, α-TEA shows the potential to inhibit DOXO-induced lung metastasis in xenograft mouse model transplanted with MDA-MB-231 cells. More in-depth studies are needed to further evaluate the combination effects of DOXO + α-TEA on the elimination of bulk as well as cancer stem cells.

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**APPENDICES**

Tiwary R, Yu W, Sanders BG, Kline K. α-TEA cooperates with chemotherapeutic agents to induce apoptosis of p53 mutant, triple-negative human breast cancer cells via activating p73. Breast Cancer Res 2011;13(1):R1. [PubMed: 21214929]

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

# α-TEA cooperates with chemotherapeutic agents to induce apoptosis of p53 mutant, triple-negative human breast cancer cells via activating p73

Richa Tiwary[1†], Weiping Yu[1*†], Bob G Sanders[1], Kimberly Kline[2]

## Abstract

**Introduction:** Successful treatment of p53 mutant, triple-negative breast cancers (TNBC) remains a daunting challenge. Doxorubicin (DOXO) and cisplatin (CDDP) are standard-of-care treatments for TNBC, but eventually fail due to acquired drug resistance and toxicity. New treatments for overcoming drug resistance and toxicity in p53 mutant, TNBC are therefore badly needed. Unlike p53, p73 - a member of the p53 family - is usually not mutated in cancers and has been shown to regulate p53-mediated apoptotic signaling in p53-deficient cancers. Therefore, identification of anticancer agents that can activate p73 in p53-deficient cancers may provide a chemotherapeutic approach for treatment of p53 mutant cancers. Here we report on the reconstitution of the p53 tumor suppressor pathway in a p53-independent manner via p73 with combination treatments of α-TEA, a small bioactive lipid, plus DOXO or CDDP.

**Methods:** p53 mutant, TNBC cell lines MDA-MB-231, BT-20 and MDA-MB-468 were used to evaluate the anticancer effect of chemotherapeutic drugs and α-TEA using annexin V (FITC)/PI staining, western blot analyses, RT-PCR and siRNA knockdown techniques.

**Results:** Combination treatments of α-TEA plus DOXO or CDDP act cooperatively to induce apoptosis, caspase-8 and caspase-9 cleavage, p73, phospho-c-Ab1 and phospho-JNK protein expression, and increase expression of p53 downstream mediators; namely, death receptor-5, CD95/APO-1 (Fas), Bax and Noxa, as well as Yap nuclear translocation - plus reduce expression of Bcl-2. Knockdown of p73, c-Abl, JNK or Yap using siRNAs shows that p73 plays a critical role in combination treatment-enhanced apoptosis and the expression of pro-apoptotic and anti-apoptotic mediators, and that c-Abl, JNK and Yap are upstream mediators of p73 in combination treatment responses.

**Conclusions:** Data show that α-TEA in combination with DOXO or CDDP synergistically enhances apoptosis in TNBC via targeting p53-mediated genes in a p73-dependent manner, and that p73 responses are downstream of c-Abl, JNK and Yap.

## Introduction

Successful treatment of triple-negative breast cancers (TNBC) (estrogen receptor (ER)-negative, progesterone receptor-negative and Her-2-negative), that are also p53 mutant remains elusive. Unfortunately, the anticancer efficacy of commonly used chemotherapeutic agents for TNBC, including doxorubicin (DOXO) and cisplatin (CDDP), are limited due to acquired drug resistance and toxicities [1,2].

DOXO and CDDP are DNA-damaging drugs that exert their anticancer actions via inhibition of cellular proliferation and induction of cell death by apoptosis [3,4]. The tumor suppressor gene p53 plays a central role in the anticancer actions of DNA-damaging agents. Loss of

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wild-type p53 functions leads to resistance to DNA-damaging agents, such as DOXO and CDDP [5,6]. Identification of anticancer agents that target p53 downstream genes via p53-independent mechanisms is of major clinical relevance, especially since p53 deficiency is a hallmark of many different cancer types.

p73 is a member of the p53 gene family [7]. Unlike p53 [8], p73 is rarely mutated or lost in cancers [9]. Although p53-deficient cancers are less responsive to chemotherapy, they are typically not completely drug resistant because other p53 family members, such as p73, can replace p53 function in response to DNA damage [9-11]. Since p73 is usually not mutated in cancers and has been shown to regulate p53 target genes in p53-deficient cancers, identification of anticancer agents that can activate p73 in p53-deficient cancers will provide a chemotherapeutic approach for treatment of drug-resistant p53 mutant cancers.

α-TEA (2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl) chroman-6-yloxyacetic acid, known as RRR-α-tocopherol ether-linked acetic acid analog or RRR-α-tocopheryloxyacetic acid) is a nonhydrolyzable ether analog of RRR-α-tocopherol [12]. α-TEA has been shown to be a potent pro-apoptotic agent both in *vitro* and *in vivo* in breast, prostate and ovarian cancer cells [12-20]. Recently, α-TEA has been shown to delay tumor onset and to inhibit the progression and metastatic spread in a clinically relevant model of spontaneous mammary cancer, further highlighting the translational potential of this anticancer agent [14]. Mechanisms involved in α-TEA-induced apoptosis include activation of JNK/c-Jun, p73/NOXA and Fas/death receptor-5 (DR5), and suppression of c-FLIP-L, survivin and phospho-Akt (pAkt) - leading to death receptor-mediated caspase-8 activation and mitochondria-dependent apoptosis [15-20].

Data presented here show that α-TEA in combination with DOXO or CDDP significantly enhances apoptosis of p53 mutant, triple-negative human breast cancer cells by targeting p73-mediated p53-dependent pro-apoptotic and anti-apoptotic genes via c-Abl, JNK and Yap signaling pathways.

## Materials and methods

### Chemicals

α-TEA was made in-house as previously described [12]. DOXO and CDDP were purchased from Sigma (San Diego, CA, USA). Phosphoinositide 3-kinase inhibitor (wortmannin) was purchased from Cell Signaling Technology (Beverly, MA, USA).

### Cell culture

p53 mutant, triple-negative human breast cancer cell lines MDA-MB-231, BT-20 and MDA-MB-468 were purchased from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 and BT-20 cells were cultured in MEM media with 10% FBS, and MDA-MB-468 cells were cultured in Dulbecco's MEM media with 10% FBS. All three p53 mutant TNBC cell lines ($ER^-$, $PR^-$, $HER2^{-/low}$) used in these studies were originally obtained from human samples so no isogenic counterparts expressing wildtype p53, ER and progesterone receptor are available for use as controls. For experiments, FBS was reduced to 2% to better mimic low *in vivo* serum exposure and cells were allowed to attach overnight before treatment. α-TEA (40 mM) was dissolved in ethanol as a stock solution. Concentrations of ethanol used in vehicle treatments were 0.025 to 0.05% (v/v) to match the ethanol content in the different final concentrations of α-TEA treatments. DOXO and CDDP were dissolved in $H_2O$.

### Quantification of apoptosis

Apoptosis was quantified by annexin V-FITC/PI assays following the manufacturer's instructions. Fluorescence was measured using fluorescence-activated cell sorter analyses with a FACSCalibur flow cytometer, and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA). Cells displaying phosphatidylserine on their surface (that is, positive for annexin-V fluorescence) were considered apoptotic.

### Nuclear and cytoplasmic fractionation

Cytoplasmic and nuclear fractions were prepared as previously described [21]. Briefly, whole cell lysates were centrifuged to obtain supernatant and pellet. The supernatant was centrifuged again and the resulting supernatant was used as the cytosolic fraction. The pellet was layered over a cushion of 1 ml sucrose buffer and centrifuged. The final pellet, lysed using RIPA buffer, was used as the nuclear fraction.

### Western blot analyses

Whole cell protein lysates were prepared and western blot analyses were conducted as described previously [22]. Proteins (20 to 50 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S-supported nitrocellulose; Schleicher and Schuell, Keene, NH, USA). Antibodies to the following proteins were used: poly(ADP-ribose) polymerase (PARP), Fas, Bcl-2, Bax, total JNK and phospho-JNK (pJNK) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p73 and NOXA (Imgenex, San-Diego, CA, USA); and pYap (Ser-127), Yap, p-cAbl (Tyr-245), c-Abl, pAkt (Ser-473), caspase-8, caspase-9, DR5 and glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology).

### RT-PCR detection of Fas, DR5, Bax, Noxa and Bcl-2 mRNA expression

Total RNA was extracted using an RNA isolation kit (Qiagen Inc., Valencia, CA, USA). Semi-quantitative analyses were conducted to detect Fas, DR5, Bax, Noxa and Bcl-2

**Table 1 Primer sequences**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| Fas | 5′-CAATGGGGATGAACCAGACTGC-3′ | 5′-GGCAAAAGAAGAAGACAAAGCC-3′ |
| DR5 | 5′-GCCTCATGGACAATGAGATAAAGGTGGCT-3′ | 5′-CCAAATCTCAAAGTACGCACAAACGG-3′ |
| Bax | 5′-AGTAACATGGAGCTGCAGAGGATG-3′ | 5′-AGGAGGCTTGAGGAGTCTCACC-3′ |
| Noxa | 5′-CGTGTGTAGTTGGCATCTCC-3′ | 5′-AAGGAGTCCCCTCATGCAAG-3′ |
| Bcl-2 | 5′-CCTGTGGATGACTGAGTACC-3′ | 5′-GAGACAGCCAGGAGAAATCA-3′ |
| β-actin | 5′-GGCGGCACCACCATGTACCCT-3″ | 5′-AGGGGCCGGACTCGTCATACT-3′ |

mRNA by RT-PCR using the housekeeping gene β-actin as control. Total RNA was reverse transcribed to cDNA using Superscript RTase (250 U; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was used per PCR reaction with Taq PCR Master Mix Kit (Qiagen Inc.) plus 10 μM oligonucleotide primer pairs (Invitrogen). The primer sequences are presented in Table 1.

### RNA interference

A scrambled RNA duplex purchased from Ambion (Austin, TX, USA) that does not target any known mouse, rat or human gene was used as the nonspecific negative control for interfering RNA (referred to as control siRNA). Transfection of MDA-MB-231 cells with siRNA to p73, c-Abl, JNK, Yap or control (Ambion) was performed in 100-mm cell culture dishes at a density of $2 \times 10^6$ cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. After 1 day of exposure to transfection mixture, the cells were re-cultured in a 100-mm dish at $2 \times 10^6$ cells/dish and incubated for 1 day followed by treatment.

### Statistical analysis

Apoptosis data were analyzed using one-way analysis of variance followed by the Tukey test for comparison of more than two treatments or a two-tailed Student $t$ test for comparison between two treatments to determine statistical differences. Differences were considered statistically significant at $P < 0.05$.

## Results

### α-TEA, DOXO and CDDP induce apoptosis in p53 mutant, human TNBC cells

The sensitivity of three p53 mutant, TNBC lines (MDA-MB-231, BT-20 and MDA-MB-468) to apoptosis induced by α-TEA, DOXO and CDDP was evaluated by determining half-maximal effective concentration values for apoptosis (Table 2). Data show that MDA-MB-468 cells exhibit the most sensitive phenotype and MDA-MB-231 cells exhibit the most resistant phenotype to apoptosis induced by DOXO and CDDP among the three cell lines. The sensitivity of the three cell lines to α-TEA-induced apoptosis, however, is similar.

### α-TEA cooperates with DOXO and CDDP to induce apoptosis of p53 mutant, TNBC cells

Based on the half-maximal effective concentration values for apoptosis presented in Table 2, the MDA-MB-231 and BT-20 cell lines that are more resistant to DOXO and CDDP were chosen to study the combinational effects of α-TEA + DOXO or α-TEA + CDDP on apoptosis induction. Data showed that α-TEA at 10 and 20 μM significantly enhanced apoptosis in combination with DOXO and CDDP in MDA-MB-231 and BT-20 cells, respectively, in comparison with individual treatments (Figure 1a to 1d). The mean combination index for the combination of α-TEA + DOXO was 0.41 ± 0.07 and 0.53 ± 0.05 for MDA-MB-231 and BT-20 cells, respectively (Table 3). The mean combination index for the combination of α-TEA + CDDP was 0.45 ± 0.10 and 0.75 ± 0.08 in MDA-MB-231 and BT-20 cells, respectively (Table 3). These data demonstrate that combinations of α-TEA + DOXO or α-TEA + CDDP synergistically induce apoptosis in both cell lines. Western blot analyses show that α-TEA at 20 μM cooperates with DOXO and CDDP to induce elevated levels of cleaved caspase-8, caspase-9, and PARP in both cell lines (Figure 2a,b), indicating that apoptosis induced by these combinations involves both caspase-8 and caspase-9 activation.

### p73 protein level is upregulated by α-TEA + DOXO or α-TEA + CDDP combinations and is involved in combination-induced apoptosis

Since DOXO and CDDP as well as α-TEA have been shown to induce p73 upregulation in breast cancer

**Table 2 Half-maximal effective concentration values for apoptosis**

| Cell line | α-TEA (μM) | DOXO (μM) | CDDP (μM) |
|---|---|---|---|
| MDA-MB-231 | 41.7 | 46.5 | 70.8 |
| BT-20 | 45.4 | 25.7 | 64.0 |
| MDA-MB-468 | 35.4 | 8.5 | 40.7 |

Cells were treated with different concentrations of α-TEA, doxorubicin (DOXO), and cisplatin (CDDP) for 24 hours. Apoptosis was determined by annexin V-FITC/PI staining/fluorescence-activated cell sorter analysis as described in Material and methods. The concentration that achieved 50% apoptosis (half-maximal effective concentration) was determined using commercially available software (Calcusyn; Biosoft, Manchester, UK).

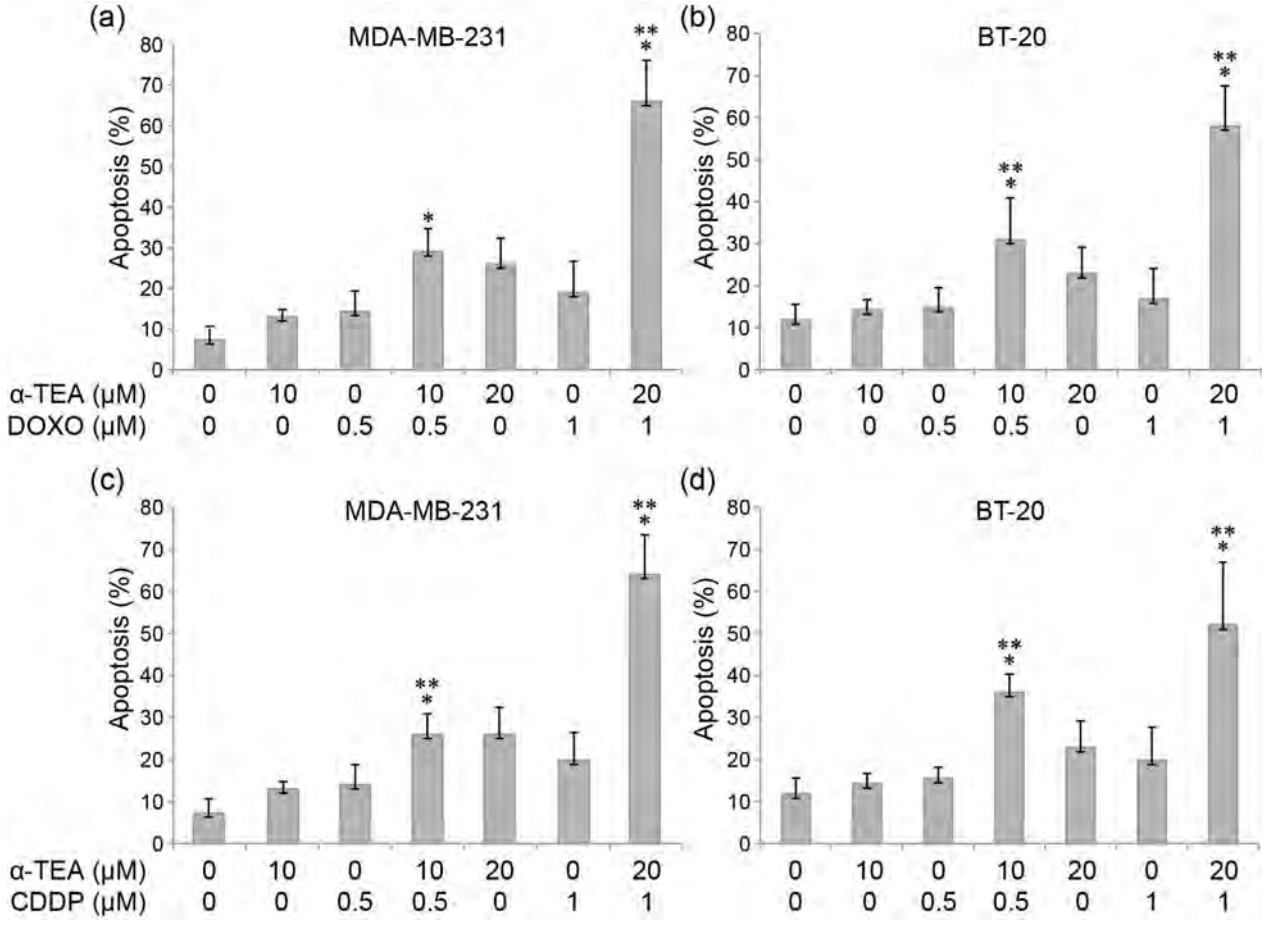


**Figure 1 α-TEA, doxorubicin and cisplatin induce apoptosis of p53 mutant, triple-negative human breast cancer cells**. MDA-MB-231 and BT-20 breast cancer cells were treated with different concentrations of doxorubicin (DOXO) or cisplatin (CDDP) alone or in combination with α-TEA for 24 hours. Fluorescence-activated cell sorter/annexin V assays were used to determine the percentage of apoptotic cells **(a), (b), (c), (d)**. Data expressed as mean ± standard deviation from three independent experiments. *$P$ <0.05, significantly different from control. **$P$ <0.05, significantly different from single treatments.

cells [11,17,23], the combination of α-TEA + DOXO or α-TEA + CDDP was investigated for ability to cooperatively enhance p73 protein expression. Single treatments with DOXO, CDDP or α-TEA at sub-apoptotic levels for 24 hours slightly increased p73 protein expression above control levels, whereas combinations at the same levels markedly enhanced p73 protein expression in comparison with single treatments in both MDA-MB-231 and BT-20 cells (Figure 3a,b). siRNA to p73 significantly reduced the ability of combination treatments to induce apoptosis as determined by annexin V (Figure 3c) and PARP analyses (Figure 3d) in MDA-MB-231 cells. Western blot data show that siRNA to p73 effectively silenced p73 protein expression (Figure 3d). These data indicate that p73 activation by combination treatments is critical for induction of cell death by apoptosis.

### Combinations of α-TEA + DOXO or α-TEA + CDDP upregulate pro-apoptotic and downregulate anti-apoptotic mediators at both mRNA and protein levels

Published data show that p73 can regulate p53-dependent genes in p53-deficient cells [11]. To better understand the cellular events involved in p73-mediated apoptosis in combination treatments, mRNA and protein expression of p53-mediated pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and anti-apoptotic mediator Bcl-2 were examined. Combinations of α-TEA + DOXO or α-TEA + CDDP enhanced DR5, Fas, Bax and Noxa mRNA (Figure 4a,b) and protein expression (Figure 4c, d), and decreased Bcl-2 mRNA (Figure 4a,b) and protein expression (Figure 4c,d) in MDA-MB-231 and BT-20 cells. siRNA knockdown of p73 was performed to determine whether expression levels of these mediators were regulated by p73. siRNA to p73 in MDA-MB-231 cells

**Table 3 Combination index of apoptosis**

| Cell line | α-TEA: drug[b] | Combination index[a] | | | | |
|---|---|---|---|---|---|---|
| | | $ED_{50}$ | $ED_{75}$ | $ED_{90}$ | Mean ± SD[c] | |
| DOXO | | | | | | |
| MDA-MB-231 | 20:1 | 0.48 | 0.40 | 0.35 | 0.41 ± 0.07 | Synergism[d] |
| BT-20 | 20:1 | 0.48 | 0.52 | 0.58 | 0.53 ± 0.05 | Synergism |
| CDDP | | | | | | |
| MDA-MB-231 | 2:1 | 0.55 | 0.44 | 0.36 | 0.45 ± 0.10 | Synergism |
| BT-20 | 4:1 | 0.68 | 0.74 | 0.84 | 0.75 ± 0.08 | Synergism |

MDA-MB-231 and BT-20 breast cancer cells were treated with different concentrations of α-TEA, doxorubicin (DOXO), and cisplatin (CDDP) alone and in combination for 24 hours. Apoptosis was determined using annexin V-FITC/PI staining/fluorescence-activated cell sorter assay as described in Material and methods. [a]For each combination treatment, a combination index was calculated using commercially available software (Calcusyn; Biosoft, Manchester, UK). [b]The ratio for the concentrations used in combination treatments was determined from the data in Figure 1. [c]The mean ± standard deviation (SD) calculated from the combination index values of the effective dose in 50% ($ED_{50}$), the effective dose in 75% ($ED_{75}$) and the effective dose in 90% ($ED_{90}$) of the population. [d]Combination index value: <1.0, synergism; 1.0, additive effect; >1.0, antagonism.

effectively silenced p73 protein expression and blocked the ability of combinations to induce increased levels of DR5, Fas, Bax and Noxa protein, as well as to decrease Bcl-2 protein levels (Figure 4e). These data suggest that combination treatments induce upregulation of pro-apoptotic mediators and downregulation of an anti-apoptotic mediator in a p73-dependent manner in p53 mutant, TNBC MDA-MB-231 and BT-20 cells. Recent studies in our laboratory show that DR5 pro-apoptotic signaling contributes to α-TEA-induced apoptosis [19,20]. To determine whether DR5 contributes to combination treatment-induced apoptosis, DR5 was functionally knocked-down with siRNA. Data indicate that silencing DR5 protein expression blocks combination-induced apoptosis as determined by PARP cleavage (Figure 4f).

### α-TEA cooperates with DOXO or CDDP to upregulate pc-Abl and pJNK, upstream mediators of p73

Studies show that p73 can be upregulated upon DNA damage via activation of c-Abl and JNK [23,24]. To understand how p73 is activated by the combination treatments, phosphorylated levels of c-Abl and JNK2/1 were examined. Combinations of α-TEA + DOXO or α-TEA + CDDP induced increased levels of pc-Abl (Tyr-245) and pJNK2/1 in both cell lines (Figure 5a,b). siRNA knockdown of c-Abl or JNK significantly reduced the ability of combination treatments to induce apoptosis in MDA-MB-231 cells as determined by annexin V (Figure 5c) and PARP cleavage (Figure 5d). siRNA treatments blocked the ability of combination treatments to increase protein levels of p73 and blocked the ability of combination treatments to increase protein levels of DR5, Fas, Bax and Noxa, and to decrease the level of Bcl-2 (Figure 5d). siRNA to c-Abl blocked the ability of combination treatments to induce increased levels of pJNK, whereas siRNA to JNK had no effect on the ability of combination treatments to induce increased levels of pc-Abl (Tyr 245) (Figure 5d). These data show that activation of p73 is mediated by c-Abl and JNK in the combination treatments, and suggest that c-Abl, in part, regulates the phosphorylation status of JNK.

### Yap is involved in combination-induced apoptosis

Since Yap, a transcriptional co-activator Yes-associated protein, can interact with p73, resulting in enhanced p73 transcriptional activity [25] and stability [26,27], we determined whether Yap contributes to combination-induced apoptosis and increased p73 expression. siRNA knockdown of Yap significantly reduced the ability of combination treatments to induce apoptosis as measured by annexin V analyses (Figure 6a) and western blot analyses of PARP cleavage (Figure 6b). siRNA to Yap effectively reduced Yap protein levels and blocked combination treatment effects on p73 protein expression, as well as combination effects on DR5, Fas, Bax, Noxa and Bcl-2 protein expression (Figure 6b). These data show that Yap is a key player in combination treatment-induced apoptosis mediated by p73.

### Combination treatments induce Yap nuclear translocation, which is associated with suppression of phosphorylation of Akt and Yap

Yap activity can be regulated by c-Abl via phosphorylation of Yap at Tyr-357, leading to its stabilization and higher affinity for p73 [28,29]. Furthermore, Yap can be negatively regulated by Akt [29,30]. Akt induces Yap phosphorylation at Ser-127, resulting in Yap cytosolic localization via promoting Yap binding with 14-3-3, resulting in inactivation of Yap [30]. Since α-TEA has been shown to decrease pAkt in prostate cancer cells [15], ovarian cancer cells [18], and breast cancer cells (data not shown) we examined the effect of combination treatments on Yap nuclear translocation, as well as on pAkt and pYap expression.

Combination treatments of MDA-MB-231 cells induced increased levels of Yap protein in the nuclear fraction and reduced levels of Yap protein in the cytoplasmic fraction. Histone 1 and glyceraldehyde-3-phosphate dehydrogenase were used to evaluate the purity of nuclear and cytoplasmic fractions, respectively, and served as lane load controls (Figure 7a). Furthermore, data show that DOXO and CDDP increased pAkt and pYap protein expression, while α-TEA cooperated with DOXO or CDDP to suppress pAkt and pYap in MDA-MB-231 (Figure 7b). These data suggest that Yap nuclear

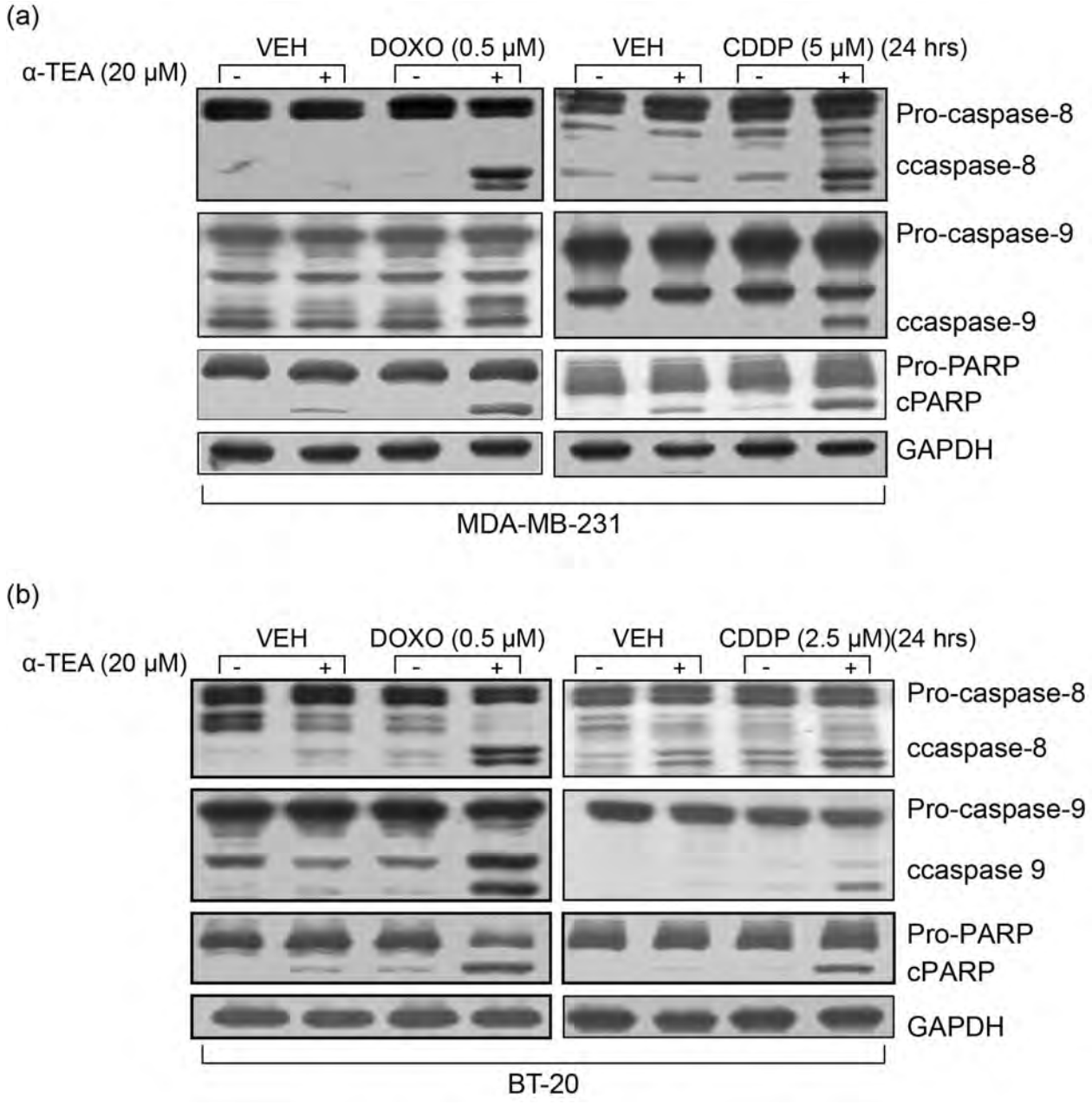


**Figure 2 α-TEA cooperates with doxorubicin and cisplatin to induce cleavage of caspase-8, caspase-9 and poly(ADP-ribose) polymerase**. MDA-MB-231 and BT-20 cells were treated with doxorubicin (DOXO) or cisplatin (CDDP) alone or in combination with α-TEA for 24 hours. Western blot analyses were used to detect poly(ADP-ribose) polymerase (PARP), caspase-8, and caspase-9 cleavage **(a), (b)**. Data are representative of at least two independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEH, vehicle.

translocation may partially contribute to p73-mediated effects and that combination treatment downregulation of pAkt correlates with decreased levels of pYap. To assess the role of Akt in DOXO-induced and CDDP-induced p73 protein expression, we examined the impact of phosphoinositide 3-kinase/Akt inhibitor (wortmannin) on DOXO-induced and CDDP-induced p73 protein expression. Data show that wortmannin enhanced DOXO-induced and CDDP-induced upregulation of p73 protein expression (Figure 7c), indicating a role for Akt in DOXO and CDDP increase in p73 expression. Data also show that wortmannin blocked DOXO-induced and CDDP-induced upregulation of pAkt and pYap (Figure 7c), suggesting that suppression of pAkt enhances DOXO-induced and CDDP-induced p73 expression via downregulation of pYap.

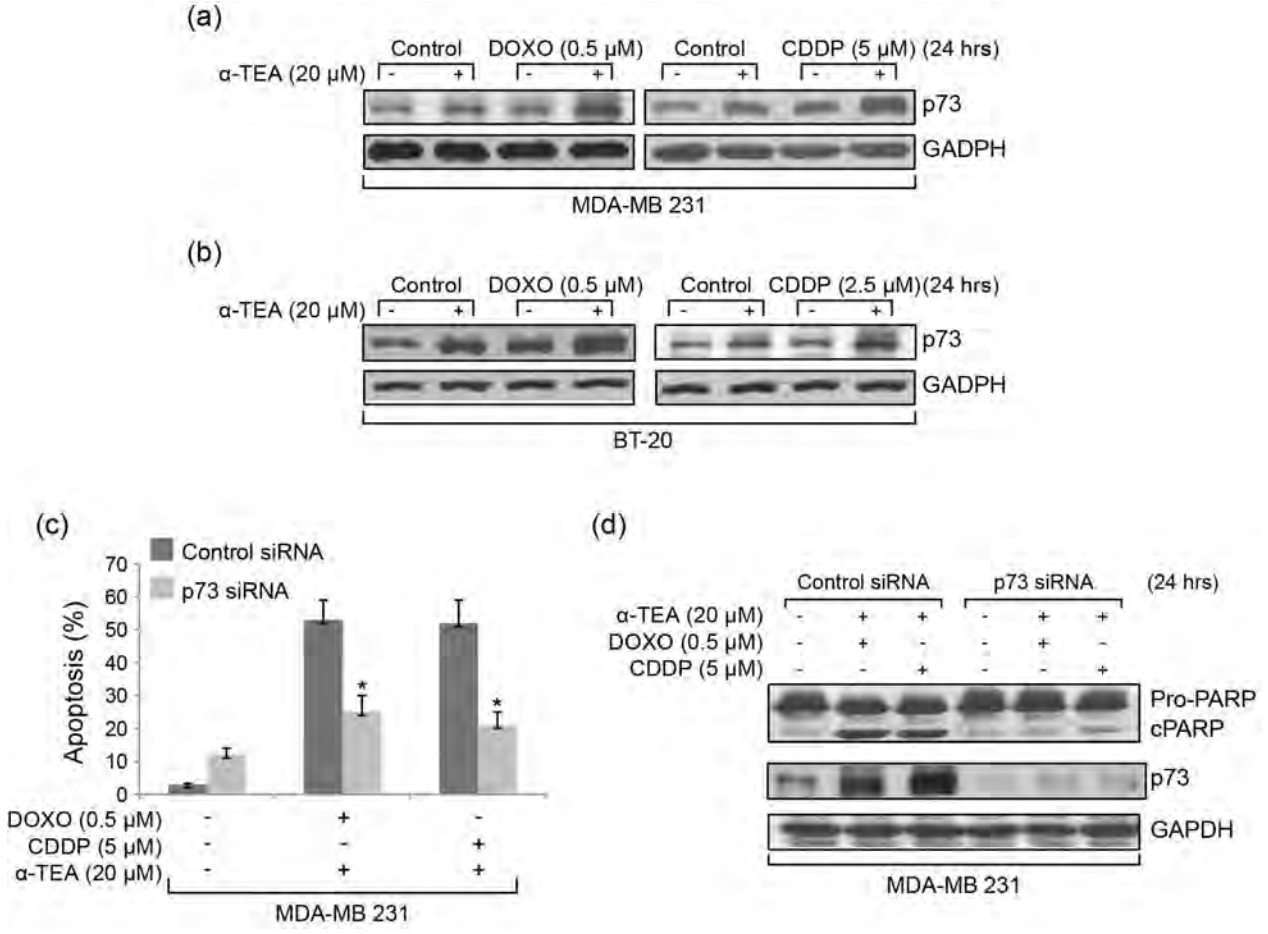


**Figure 3 p73 is upregulated by combination treatments and involved in combination-induced apoptosis**. MDA-MB-231 and BT-20 cells were treated with α-TEA, doxorubicin (DOXO) and cisplatin (CDDP) alone or in combination for 24 hours. Western blot analyses were performed to detect protein levels of p73 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as loading control **(a), (b)**. MDA-MB-231 cells were transfected with p73 siRNA or control siRNA for 2 days and treated with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hours. Fluorescence-activated cell sorter/annexin V assays were used to determine the percentage of apoptotic cells **(c)**. Western blot analyses were used to verify the knockdown efficiency of p73 siRNA and the effect of p73 siRNA on combination-induced poly(ADP-ribose) polymerase (PARP) cleavage **(d)**. Data in (a), (b) and (d) representative of at least two independent experiments; data in (c) expressed as mean ± standard deviation from three independent experiments. *$P < 0.05$, significantly different from control siRNA determined by *t* test.

## Discussion

p73 is an important target for treating p53 mutant cancers [10,31-33]. The novel findings in the present study are as follows. First, α-TEA - a potent anticancer analog of vitamin E - synergizes with DNA-damaging agents DOXO and CDDP to induce apoptosis of human p53 mutant, triple-negative human breast cancer MDA-MB-231 and BT-20 cells via targeting p73. Second, combination treatments result in p73-dependent upregulation of pro-apoptotic DR5, Fas, Bax and Noxa, and downregulation of anti-apoptotic mediator Bcl-2 - all of which are p53-mediated apoptotic-related genes. Third, p73 and p73-mediated apoptotic events are regulated by c-Abl, JNK and Yap in combination treatments. Finally, α-TEA downregulation of Akt partially contributes to p73 upregulation in combination treatments. Our data therefore, for the first time, identify α-TEA as a small bioactive anticancer agent that regulates p53-mediated genes via p53-independent mechanisms when combined with DNA-damaging agents.

As a transcription factor, p73 shares structural and functional similarities with p53 [9,32,33]. In cancer cells that express wildtype p53, p73 has been reported to cooperate with p53 to induce apoptosis [34]; whereas in p53 mutant cancer cells, p73 has been reported to induce apoptosis via activation of p53-inducible genes [11,35]. Typically, p53 induces apoptosis via regulating apoptosis-related genes such as DR5, Fas, Bax, Noxa and Bcl-2 [36,37]. p73 is upregulated in response to a subset of DNA-damaging agents, including DOXO,

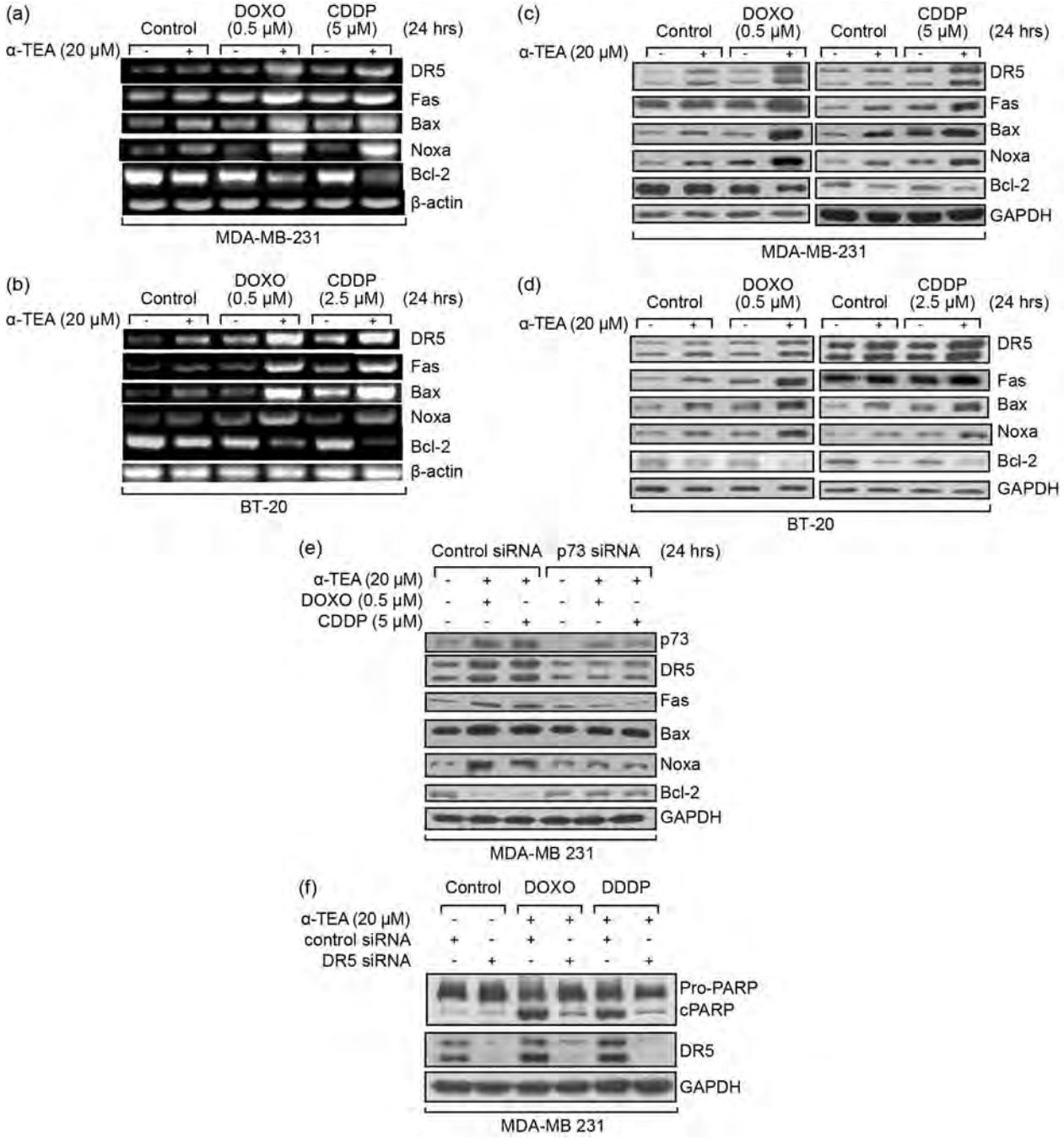


**Figure 4 α-TEA cooperates with doxorubicin or cisplatin to upregulate pro-apoptotic and downregulate anti-apoptotic mRNAs and proteins**. α-TEA cooperates with doxorubicin (DOXO) or cisplatin (CDDP) to upregulate mRNAs and proteins of the pro-apoptotic mediators death receptor 5 (DR5), Fas, Bax, and Noxa, and downregulate anti-apoptotic Bcl-2 mRNA and protein, all of which are downstream targets of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hours. mRNA levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by RT-PCR with β-actin serving as loading control **(a), (b)**. Protein levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by western blot analyses with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as loading control **(c), (d)**. The same treated samples as Figure 3d were used to detect the effect of siRNA to p73 on the combination-induced increase in protein levels of DR5, Fas, Bax, Noxa and decrease in Bcl-2 by western blot analyses with GAPDH as loading control **(e)**. MDA-MB-231 cells were transfected with DR5 siRNA or control siRNA for 2 days and treated with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hours. Western blot analyses were used to determine the effect of siRNA to DR5 on combination-induced poly(ADP-ribose) polymerase (PARP) cleavage and to verify the knockdown efficiency of DR5 **(f)**. Data representative of at least two independent experiments.

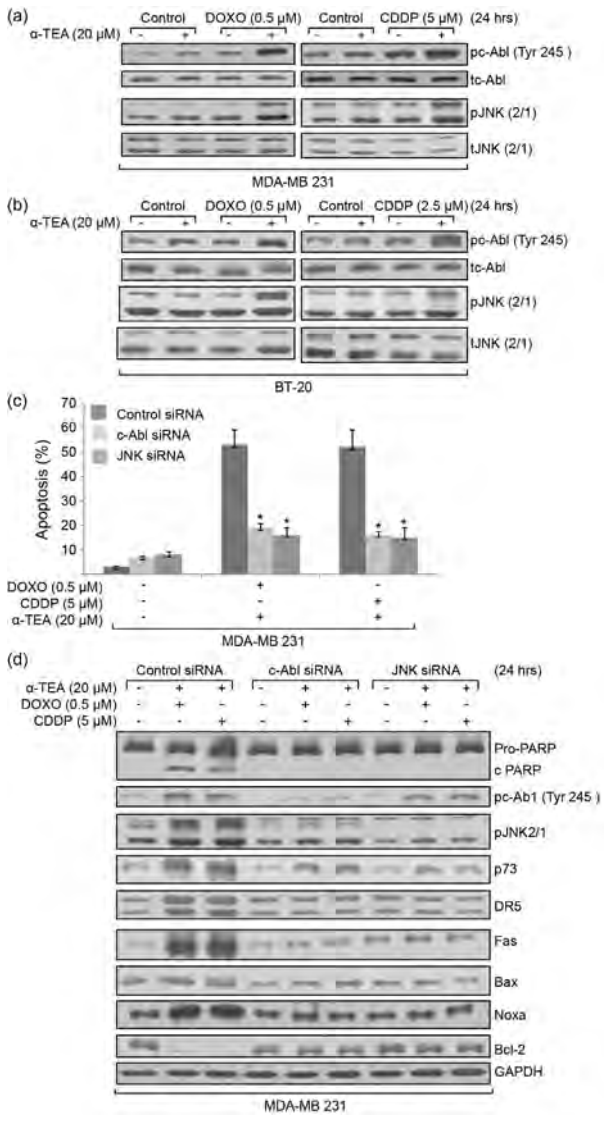


**Figure 5 α-TEA cooperates with doxorubicin or cisplatin to upregulate pc-Abl and pJNK, which can serve as upstream mediators of p73**. α-TEA cooperates with doxorubicin (DOXO) or cisplatin (CDDP) to upregulate pc-Abl and pJNK, which can serve as upstream mediators of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CIDDP alone or in combination with α-TEA for 24 hours. Protein levels of pc-Abl (Tyr-245), total c-Abl (tc-Abl), pJNK2/1, and total JNK2/1 (tJNK2/1) were determined by western blot **(a), (b)**. MDA-MB-231 cells were transfected with c-Abl and JNK siRNAs, as well as control siRNA for 2 days and treated with a combination of α-TEA + DOXO or α-TEA + CDDP for 24 hours. Apoptosis was determined by annexin V/fluorescence-activated cell sorter **(c)**. Western blot analyses were used to verify the knockdown efficiency of c-Abl and JNK siRNAs and the effect of c-Abl and JNK siRNAs on combination-induced poly(ADP-ribose) polymerase (PARP) cleavage, as well as p73 and p73-mediated death receptor 5 (DR5), Fas, Bax, Noxa and Bcl-2 **(d)**. Data in (a), (b), and (d) representative of at least two independent experiments; data in (c) expressed as mean ± standard deviation from three independent experiments. *$P$ <0.05, significantly different from control siRNA determined by $t$ test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

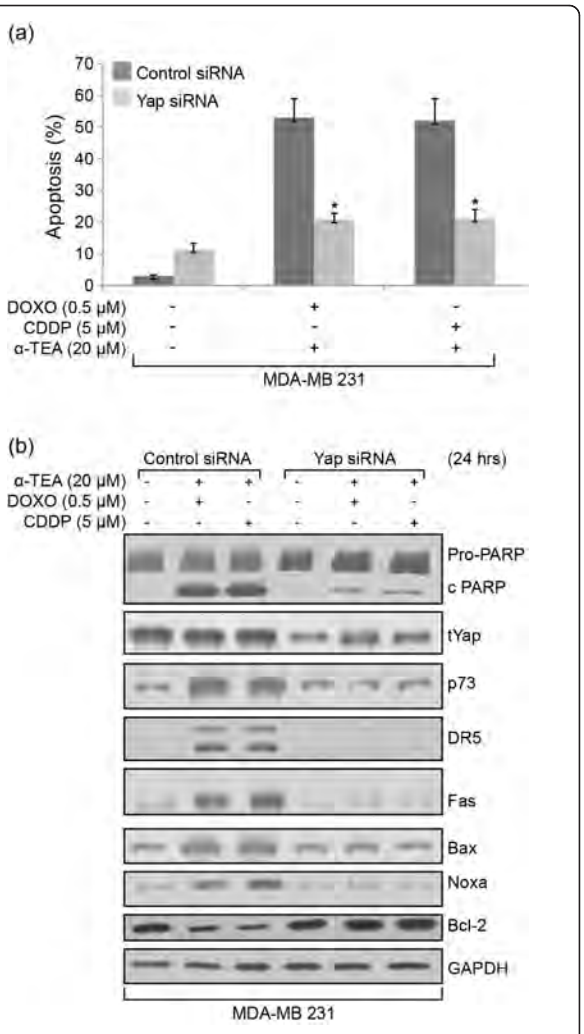


**Figure 6 Yap is involved in combination treatment-induced apoptosis**. MDA-MB-231 cells were transfected with Yap siRNA or control siRNA for 2 days and treated with combinations for 24 hours. Apoptosis was determined by annexin V/fluorescence-activated cell sorter **(a)**. Western blot analyses were used to verify the knockdown efficiency of Yap siRNA and the effect of Yap siRNA on combination-induced poly(ADP-ribose) polymerase (PARP) cleavage, as well as p73 and p73-mediated death receptor 5 (DR5), Fas, Bax, Noxa and Bcl-2 **(b)**. Data (a) expressed as mean ± standard deviation from three independent experiments; data in (b) representative of at least two independent experiments. *$P$ <0.05, significantly different from control siRNA determined by $t$ test. CDDP, cisplatin; DOXO, doxorubicin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CDDP, camptothecin and etoposide [38]. Several p53-mediated apoptosis-related genes have been identified to be regulated by p73, such as Fas, Bax, Bim, Noxa and Puma [17,39-41]. Whether DR5 is a direct target of p73, however, is not well documented. It has been reported that DR5 is regulated by p73 in H1299 human nonsmall lung cancer cells [42]. El-Deiry and coworkers used a high-throughput screen to identify small molecules that

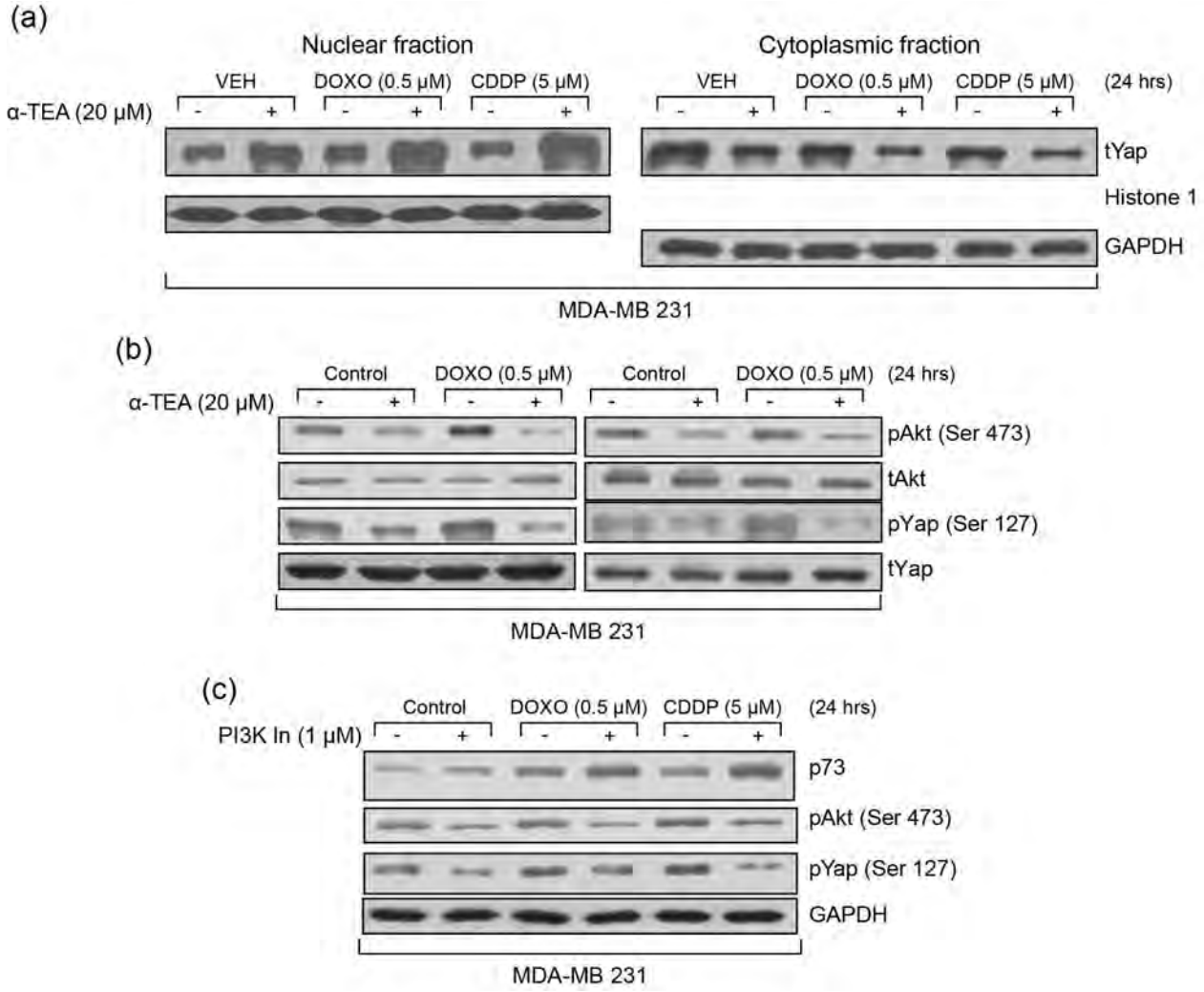


**Figure 7 Combination treatments induce Yap nuclear translocation, associated with suppression of phosphorylation of Akt and Yap**. Isolated cytosolic and nuclear fractions from MDA-MB-231 cells treated with α-TEA + doxorubicin (DOXO) or α-TEA + cisplatin (CDDP) were used to detect Yap tanslocation from cytosol to the nucleus by western blot analyses **(a)**. MDA-MB-231 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hours. Protein levels of pAkt and pYap were determined by western blot analyses with total Akt and Yap serving as controls **(b)**. MDA-MB-231 cells were pre-treated with Akt inhibitor wortmannin at 1 μM or dimethylsulfoxide for 2 hours followed by treatments with DOXO or CDDP for 24 hours. Protein levels of p73, pAkt and pYap were determined by western blot analyses **(c)**. Data are representative of at least two independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEH, vehicle.

could activate p53 reporter activity, increase expression of p53 target genes such as p21(Waf1), DR5 and TRAIL, and induce apoptosis in p53-deficient colon cancer cells [35]. Some of these compounds activated a p53 response by increasing p73 expression, and knockdown of p73 with siRNA reduced their ability to activate p53 reporter activity while other compounds acted in a p73-independent fashion [35]. In addition, they characterized a derivative of the plant alkaloid ellipticine as an anticancer agent that induces p73 and DR5 protein expression in a p53-deficient human colon carcinoma cell line [43]. Neither of these studies, however, showed direct evidence that p73 was regulating DR5 transcription. To the best of our knowledge, there is no direct evidence showing that p73 regulates DR5 transcription other than the lung cancer studies [42]. In addition, there is no evidence to indicate that p73 transcriptionally regulates Bcl-2. The present study thus demonstrates, for the first time, that both DR5 and Bcl-2 are mediated at the transcriptional level by p73 in p53 mutant, TNBC MDA-MB-231 and BT-20 human breast cancer cells treated with α-TEA combined with DOXO or CDDP as determined by siRNA knockdown assays. Our previous data showed that DR5 is involved in α-TEA-induced apoptosis since siRNA knockdown of DR5 blocked α-TEA-induced apoptosis in MCF-7 and MDA-MB-231 human breast

cancer cells [19,20]. Here, we demonstrated that DR5 is necessary, at least in part, for apoptosis induced by α-TEA combination treatments with DOXO or CDDP.

Besides transcriptionally activating p53-mediated apoptotic genes, p73 has been reported to induce ER stress via transactivation of Scotin [44]. Since DR5 and Bcl-2 expression can be regulated by ER stress via CHOP [45,46], and since α-TEA has been shown to induce ER stress and CHOP expression [19], we cannot rule out the possibility that p73 regulates DR5 and Bcl-2 via ER stress in combination treatments. Further studies are needed to address this issue.

p73 is predominantly regulated at the post-translational level in response to DNA-damaging agents. c-Abl and JNK are activated by DNA-damaging agents and both are involved in p73 activation [23,24]. DOXO and CDDP have been shown to regulate p73 via c-Ab1 [23,28,29]. c-Abl regulates p73 via different mechanisms; for example, c-Abl can directly stabilize p73 via acetylation and phosphorylation of p73 [23,47], and can stabilize p73 and enhance p73 transcriptional activity via phosphorylation of Yap [28]. JNK has been reported to stabilize p73 via phosphorylation of p73 [24] and via JNK phosphorylation/activation of c-Jun [48]. In addition, JNK also activates p73 via enhancing c-Abl nuclear translocation [49]. In untreated cells, c-Abl is sequestered in the cytosol by 14-3-3 proteins. Upon exposure of cells to DNA damaging agents, JNK is activated and phosphorylates 14-3-3, resulting in the release of c-Abl into the nucleus, an event required for the induction of apoptosis in response to DNA-damaging agents [49]. Published data [50] and the present data show that c-Abl also regulates JNK via phosphorylation, suggesting cross-talk between c-Abl and JNK.

Yap is a transcriptional coactivator, which can interact with the p53 family member p73, resulting in an enhancement of p73's transcriptional activity [25,26] and stability [27]. A potential mechanism of the p73 protein stabilization was recently suggested by Levy and colleagues [27]. Namely, Yap competes with Itch, an E3 ubiquitin ligase involved in degradation of p73, for binding to p73 at the PPXY motif. Furthermore, Yap activity can be regulated by c-Abl via phosphorylation at Tyr-357, leading to a more stable form of Yap that exhibits a higher affinity to p73 [27]. Yap can be negatively regulated by Akt [30,51]. Akt induces Yap phosphorylation at Ser-127, resulting in Yap cytosolic localization since phosphorylation of Yap at Ser-127 promotes Yap binding with 14-3-3 [30]. Yap activation can thus be regulated in a positive manner by c-Abl and in a negative manner by Akt. DNA damage can activate survival mediator Akt, resulting in reducing the anticancer efficacy of DNA-damaging drugs. DOXO or CDDP induces activation of Akt in some cell lines [52,53]. Likewise, our data show that DOXO and CDDP induce elevated levels of pAkt not only in MDA-MB-231 cells (Figure 7), but also in MCF-7, MDA-MB-453 and BT-20 cells (data not shown). As expected, Akt inhibitors have been reported to enhance the anticancer effect of DOXO in MDA-MB-231 cells [54]. Data reported here show that Akt inhibitor wortmannin enhanced DOXO-mediated and CDDP-mediated increases in p73 protein expression, which is associated with downregulation of pAkt and pYap (Ser-127) in MDA-MB-231 cells. Taken together, these data suggest that Akt activation upon DNA damage may counteract p73 activation induced by JNK and c-Abl via inhibition of Yap nuclear translocation. Our data thus suggest that Yap nuclear translocation plays an important role in p73 activation and that suppression of pAkt and its inhibitory phosphorylation of pYap contributes to enhanced Yap nuclear translocation in combination treatments.

How α-TEA induces p73 protein expression is not fully understood. We previously reported that JNK is involved in regulation of p73 in α-TEA-induced apoptosis of human breast cancer cells [19]. In the present study, we found that α-TEA also induces increased levels of pc-Abl and Yap nuclear translocation, as well as suppresses pAkt and pYap, suggesting that c-Abl and Yap, as well as downregulation of pAkt/pYap, are also involved in α-TEA-induced apoptosis. Noxa has been identified as a downstream mediator of p73 in α-TEA-induced apoptosis [17]. Whether other p53-mediated genes, such as Fas, DR5, Bax and Bcl-2, are regulated by 73 following α-TEA treatment, however, has not been investigated. Since recent data show that ER stress-mediated CHOP contributes to α-TEA-induced upregulation of DR5 and downregulation of Bcl-2 [19], it will be important for future studies to address whether both CHOP and p73 contribute to DR5 upregulation and Bcl-2 downregulation in α-TEA-induced apoptosis.

Mechanisms mediating the combined anticancer effects of α-TEA + DOXO or α-TEA + CDDP are diverse and not completely understood. These studies identified p73 as a key player in combination treatment-induced apoptosis. In addition, data show that c-Abl, JNK and Yap play roles in combination treatment-induced activation of p73. It is important to note that although both α-TEA and DNA-damaging drugs DOXO or CDDP induce increased levels of pc-Ab1 and pJNK, only α-TEA and the combination of α-TEA + DOXO or α-TEA + CDDP induce Yap nuclear translocation, which is associated with inhibition of pAkt (Ser-473) and Akt-phosphorylated pYap (Ser-127). Furthermore, a phosphoinositide 3-kinase/Akt inhibitor was shown to enhance DOXO and CDDP upregulation of p73, which was also associated with downregulation of pAkt and pYap. Taken together, these data suggest that downregulation of pAkt and the pAkt-mediated inactive form of Yap play important roles

in p73 activation and apoptosis in combination treatments. α-TEA thus cooperates with DOXO or CDDP to induce p73 protein expression and apoptosis not only via activation of c-Abl and JNK, but also via activation of Yap, which may be regulated positively by c-Abl and negatively by Akt. Based on published reports and the data presented here we proposed signaling events necessary for combination treatment-induced apoptosis in p53 mutant, TNBC cells (Figure 8).

## Conclusions

In summary, the data demonstrate that α-TEA, a small bioactive lipid, cooperates with DNA-damaging agents DOXO and CDDP to induce apoptosis in human breast cancer cells via targeting p53-inducible apoptotic-related genes in a p73-dependent manner. These studies highlight the potential for activation of p73 as a promising target for treatment of p53 mutant, TNBC and identify α-TEA as an important candidate agent.

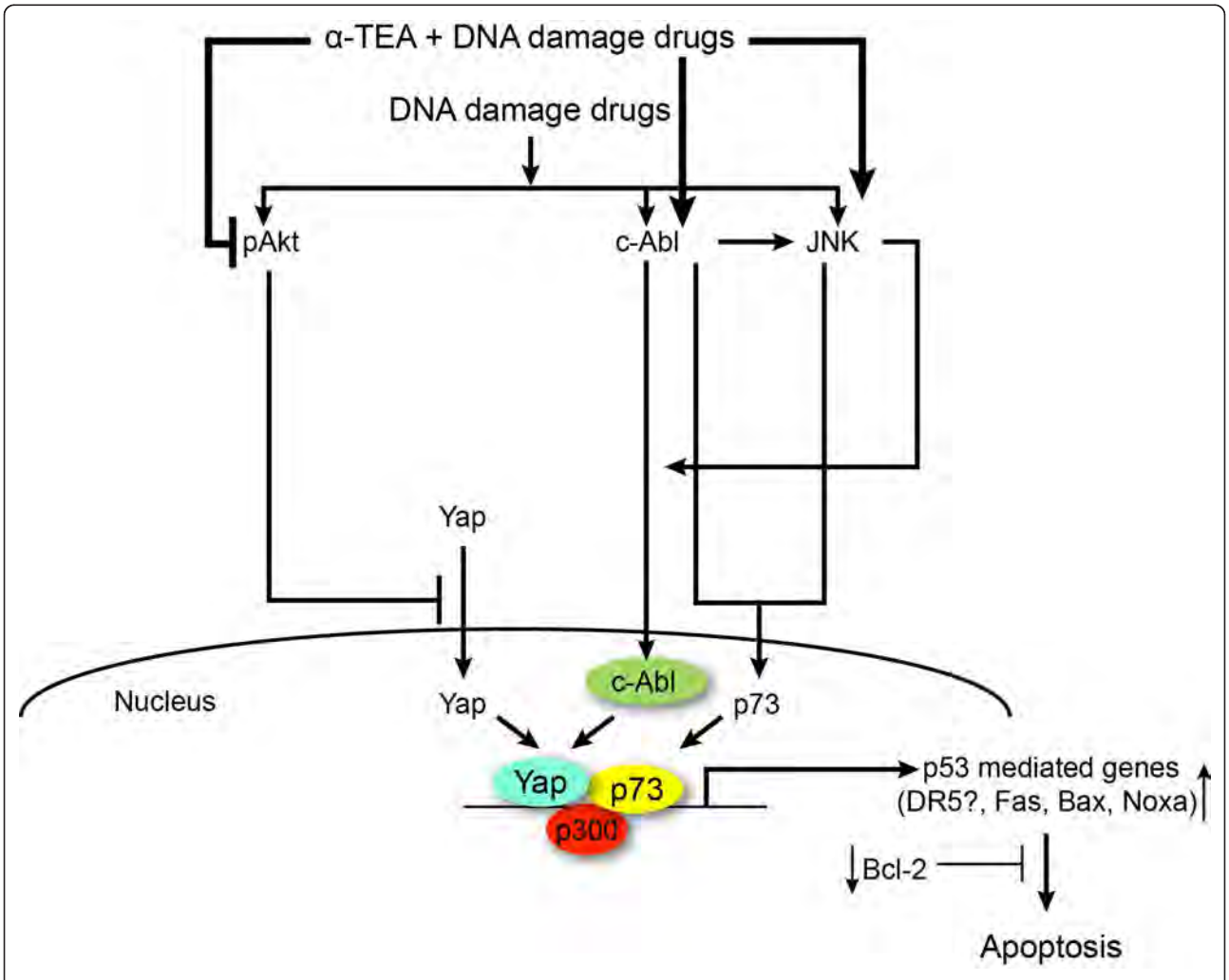


**Figure 8 Proposed signaling pathways**. Proposed pathways whereby the combination of α-TEA + doxorubicin (DOXO) or α-TEA + cisplatin (CDDP) induces apoptosis in p53 mutant, triple-negative MDA-MB-231 and BT-20 human breast cancer cells. p73 can be activated via multiple mechanisms and pathways, including: c-Abl and JNK can directly phosphorylate p73 to stabilize it; Yap in nucleus can bind with p73 to enhance its transcriptional activity and stability; and p73 can be transcriptionally regulated (not studied here). Yap can be regulated in a positive manner by c-Abl via phosphorylation enhancing its stability and transcriptional activity, and in a negative manner by Akt via inhibiting translocation of Yap into the nucleus. c-Abl can directly phosphorylate JNK and JNK can enhance c-Abl nuclear translocation. Therefore, c-Abl, JNK and Yap play positive roles and Akt plays a negative role in p73 activation. Our data show that DNA-damaging drugs DOXO and CDDP activated c-Abl and JNK, but also activated Akt, which can counteract c-Abl and JNK effects on activation of p73. Combination treatments not only act cooperatively to activate c-Abl and JNK, but also act cooperatively to inhibit pAkt and pYap (ser-127), leading to Yap nuclear translocation and p73 activation. In summary, combinations of α-TEA + DOXO or α-TEA + CDDP act cooperatively to upregulate c-Abl/JNK, induce Yap nuclear translocation and downregulate pAkt/pYap, leading to activation of p73 and upregulation of p73-mediated pro-apoptotic factors mediators, and downregulation of Bcl-2, thereby restoring DOXO and CDDP chemotherapeutic potential in p53 mutant, triple-negative breast cancers. DR5, death receptor 5.

**Abbreviations**

α-TEA: RRR-α-tocopherol ether-linked acetic acid analog; CDDP: cisplatin; DOXO: doxorubicin; DR5: death receptor 5; ER: estrogen receptor; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; JNK: c-Jun N-terminal kinase; MEM: modified Eagle's medium; p-Akt: phospho-Akt; PARP: poly(ADP-ribose) polymerase; PCR: polymerase chain reaction; PI: propidium iodide; pJNK: phospho-c-Jun N-terminal kinase; RT: reverse transcriptase; siRNA: small interfering RNA; TNBC: triple-negative breast cancers; Yap: Yes-associated protein.

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**Authors' contributions**

RT, WY, BGS and KK conceived and designed the study, analyzed the data, and drafted the manuscript. WY helped in cell culture study and RT performed all experiments.

**Competing interests**

US and international patents on α-TEA are held by the Research Development Foundation. KK, BGS and WY are listed as inventors. No commercial applications or financial gain have been realized.

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